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Index

F a s c. 1

	Page
BOLLE-JONES, E. W.: Tentative method of sugar estimation in laminae and its application to <i>Hevea brasiliensis</i>	1
ASANA, R. D. and MANI, V. S.: Studies in physiological analysis of yield. II. Further observations on varietal differences in photosynthesis in leaf, stem and ear of wheat	8
SIEGEL, S. M.: The biochemistry of lignin formation	20
EVENARI, M., NEUMANN, G., and KLEIN, S.: The influence of red and infrared light on the respiration of photoblastic seeds	33
STREET, H. E.: Factors controlling meristematic activity in excised roots VI. Effects of various 'antiauxins' on the growth and survival of excised roots of <i>Lycopersicum esculentum</i> , Mill.	48
HATTORI, and SHIROYA, M.: Studies on the browning and blackening of plant tissues. II. On the interaction of dopa and a specific oxidase in the leaves of <i>Stizolobium Hassjo</i>	63
WAI, N.: Effects of some antiseptics on the growth of <i>Chlorella</i>	71
FREDRICK, J. F. and MULLIGAN, F. J. JR.: Mechanism of action of branching enzyme from <i>Oscillatoria</i> and the structure of branched dextrins	74
LUNDEGÅRDH, H.: Spectrophotometrical determinations of peroxidase in living roots	84
LUNDEGÅRDH, H.: On partial oxidation of the cytochrome system in the presence of cyanide	95
STEEMANN NIELSEN, E.: Influence of pH on the respiration in <i>Chlorella pyrenoidosa</i>	106
GHOSE, T. K. and WIKÉN, T.: Inhibition of bacterial sulphate-reduction in presence of short chain fatty acids	116
GUNDERSEN, K.: Effects of B-vitamins and amino-acids on nitrification	136
LUNDEGÅRDH, H.: On the cytochromes b and dh in the roots of cereals	142
FRIES, N.: Further experiments on the response of decotylised pea seedlings to arginine	164
BURSTRÖM, H.: Evaluation of the growth activity of naphthalene derivatives ..	174
CANTINO, E. C. and HORENSTEIN, E. A.: The role of ketoglutarate and polyphenol oxidase in the synthesis of melanin during morphogenesis in <i>Blastocladiella emersonii</i>	189

F a s c. 2

YOUNIS, A. F.: Studies on the photoperiodism of <i>Kalanchoë Blossfeldiana</i> I. Effect of age on response to short-day treatment	223
YOUNIS, A. F.: Studies on the photoperiodism of <i>Kalanchoë Blossfeldiana</i> II. A. Flowering of Dutch variety. B. Flowering of cuttings under short-days	230

ROBERTS, E. H. and STREET, H. E.: The continous culture of excised rye roots	238
SCHWABE, W. W.: Photoperiodic cycles of lengths differing from 24 hours in relation to endogenous rhythms	263
ASANA, R D., MANI, V. S. and VEDPRAKASH: The effect of auxins on the growth and yield of wheat	279
FREDRICK, J. F.: Proposed molecular structure for straight and branched polymers of glucose	288
GENTILE, A. C. and KLEIN, R. M.: The apparent necessity of indoleacetic acid for the growth of <i>Diplodia</i> (<i>Fungi imperfecti</i>)	291
BLACK, M. and WAREING, P. F.: Growth studies in woody species VII. Photoperiodic control of germination in <i>Betula pubescens</i> Ehrh.	300
STEEMANN NIELSEN, E.: Carbon dioxide as carbon source and narcotic in photosynthesis and growth of <i>Chlorella pyrenoidosa</i>	317
FRANSSON, P. and INGESTAD, T.: The effect of an antiauxin on the indoleacetic acid content in <i>Avena coleoptiles</i>	336
LARSEN, P.: On the separation of acidic and non-acidic auxins	343
SHIROYA, M. and HATTORI, S.: Studies on the browning and blackening of plant tissues. III. Occurrence in the leaves of <i>Dahlia</i> and several other plants of chlorogenic acid as the principle browning agent	358
SCHAFFALITZKY DE MUCKADELL, M.: A development stage in <i>Fagus silvatica</i> characterized by abundant flowering	370
ELIASSON, L.: The connection between the respiratory gradient and the growth rate in wheat roots	374
VIRGIN, H. I.: The conversion of protochlorophyll to chlorophyll a in continuous and intermittent light	389
ANDERSEN, S.: Relation between leaf number and ear development in spring-sown barley and oats	404
HEMBERG, T.: Studies on the balance between free and bound auxin in germinating maize	418
HYLMÖ, B.: Passive components in the ion absorption of the plant. I. The zonal ion and water absorption in Brouwer's experiments	433

F a s c. 3

Dedication to Professor OSCAR HAGEM	451
MOYSE, A.: Le métabolisme des acides organiques chez le <i>Bryophyllum</i> (Crassulacée) I. Oxydations respiratoires et fixation de l'anhydride carbonique à l'obscurité par β -carboxylation, en fonction de la tension d'oxygène	453
MOYSE, A.: Le métabolisme des acides organiques chez le <i>Bryophyllum</i> (Crassulacée) II. Les variations de l'acidité et la photosynthèse, en fonction de la tension d'oxygène	478
HULTGREN, B., KIHLMAN, B., and FRIES, N.: Antagonists to the caffeine-inhibition of fungal growth	493
HALVORSEN, H.: The gas exchange of flax seeds in relation to temperature I. Experiments with immature seeds and capsules	501
CARR, D. J.: On the nature of photoperiodic induction III. The summation of the effects of inductive photoperiodic cycles	512

MASUDA, Y.: Der Einfluss des Heteroauxins auf die Plasmapermeabilität für Harnstoff und Alkylharnstoffe	527
ARNON, D. I., ICHIOKA, P. S., WESSEL, G., FUJIWARA, A., and WOOLLEY, J. T.: Molybdenum in relation to nitrogen metabolism I. Assimilation of nitrate nitrogen by <i>Scenedesmus</i>	538
ICHIOKA, P. S. and ARNON, D. I.: Molybdenum in relation to nitrogen metabolism II. Assimilation of ammonia and urea without molybdenum by <i>Scenedesmus</i>	552
KARLSSON, B. and ELIASSON, L.: The respiratory quotient in different parts of wheat roots in relation to growth	561
STÅLFELT, M. G.: The stomata as a hydrophotic regulator of the water deficit of the plant	572
SHIROYA, M., SHIROYA, T., and HATTORI, S.: Studies on the browning and blackening of plant tissues IV. Chlorogenic acid in the leaves of <i>Nicotiana tabacum</i>	594
BOLLE-JONES, E. W.: Comparative effects of ammonium and nitrate ions on the growth and composition of <i>Hevea brasiliensis</i>	606
VIRGIN, H. I.: Protochlorophyll formation and greening in etiolated barley leaves	630
GYLLENBERG, H.: The »rhizosphere effect» of graminaceous plants in virgin soils	644
ALLEN, M. B. and ARNON, D. I.: Studies on nitrogen-fixing blue-green algae II. The sodium requirement of <i>Anabaena cylindrica</i>	653
DAHL, L.-G.: The influence of some inorganic salts on the sporulation of a strain of <i>Bacillus stearothermophilus</i>	661
BRIAN, P. W. and HEMMING, H. G.: The effect of gibberellic acid on shoot growth of pea seedlings	669
GENTILE, A. C. and NAYLOR, A. W.: The metabolism of <i>Rumex virus</i> tumors. Terminal respiratory enzymes	682
NORDBRING-HERTZ, B.: Studies on growth and inhibition of <i>Candida albicans</i>	691

F a s c. 4

GOKSØYR, JOSTEIN: The effect of some dithiocarbamyl compounds on the metabolism of fungi	719
KROG, JOHN: Notes on temperature measurements indicative of special organization in arctic and subarctic plants for utilization of radiated heat from the sun	836
JØRGENSEN, ERIK G.: Variations in the silica content of diatoms	840
— Solubility of the silica in diatoms	846
FRIES, NILS and YUSEF, HASAN M.: Two fatty acid requiring mutants of <i>Ophiostoma multiannulatum</i>	852
FRIES, NILS: The significance of thiamin and pyridoxin for the growth of the decotylised pea seedling	859
KRALL, ALBERT R.: Cytochrome oxidase participation in photosynthetic fixation of carbon dioxide: specific light reversal of carbon monoxide inhibition ..	869
FARKAS, G. L. and KIRÁLY, Z.: Studies on the respiration of wheat infected with stem rust and powdery mildew	877
PILET, P.-E. and GALSTON, A. W.: Auxin destruction, peroxidase activity, and peroxide genesis in the roots of <i>Lens culinaris</i>	888
BRIAN, P. W., HEMMING, H. G. and RADLEY, MARGARET: A physiological comparison of gibberellic acid with some auxins	899

BONDE, ERIK K.: The effect of various cycles of light and darkness on the growth of tomato and cocklebur plants	913
SYRETT, P. J.: The assimilation of ammonia and nitrate by nitrogen-starved cells of <i>Chlorella vulgaris</i> I. The assimilation of small quantitatives of nitrogen	924
MEIDNER, HANS: The determination of paths of air movement in leaves	930
FREDRICK, JEROME F. and MANCINI, ARMANDO F.: Paper electrophoresis patterns of enzymes involved in polyglucoside synthesis in <i>Oscillatoria princeps</i> and its low temperature strains	936
STEEMANN NIELSEN, E.: The interaction of photosynthesis and respiration and its importance for the determination of ^{14}C -discrimination in photosynthesis	945
VIRGIN, HEMMING I.: A new method for the determination of the turgor of plant tissues	954
STOY, V.: Action of different light qualities on simultaneous photosynthesis and nitrate assimilation in wheat leaves	963
HANSEN, B. A. M., BURSTRÖM, H., and TEÄR, J.: Root and shoot elongation activity of some naphthalene compounds	987

Index auctorum

	Page		Page
Allen, M. B.	653	Klein, R. M.	291
Andersen, S.	404	Klein, S.	33
Arnon, D. I.	538, 552, 653	Krall, A. R.	869
Asana, R. D.	8, 279	Krog, J.	836
Black, M.	300	Larsen, P.	343
Bolle-Jones, E. W.	1, 606	Lundegårdh, H.	84, 95, 142
Bonde, E. K.	913	Mancini, A. F.	936
Brian, P. W.	669, 899	Mani, V. S.	8, 279
Burström, H.	174, 987	Masuda, Y.	527
Cantino, E. C.	189	Meidner, H.	930
Carr, D. J.	512	Moyse, A.	453, 478
Dahl, L.-G.	661	Mulligan, F. J. Jr.	74
Eliasson, L.	374, 561	Naylor, A. W.	682
Evenari, M.	33	Neumann, G.	33
Farkas, G. L.	877	Nordbring-Hertz, B.	691
Fransson, P.	336	Pilet, P.-E.	888
Fredrick, J. F.	74, 288, 936	Radley, M.	899
Fries, N.	164: 493, 852, 859	Roberts, E. H.	238
Fujiwara, A.	538	Schaffalitzky de Muckadell, M.	370
Galston, A. W.	888	Schwabe, W. W.	263
Gentile, A. C.	291, 682	Shiroya, M.	63, 358, 594
Ghose, T. K.	116	Shiroya, T.	594
Goksoyr, J.	719	Siegel, S. M.	20
Gundersen, K.	136	Stålfelt, M. G.	572
Gyllenberg, H.	644	Steemann Nielsen, E.	106, 317, 945
Halvorsen, H.	501	Stoy, V.	936
Hansen, B. A. M.	987	Street, H. E.	48, 238
Hattori, S.	53, 358, 594	Syrett, P. J.	924
Hemberg, T.	418	Teär, J.	987
Hemming, H. G.	669, 899	Vedprakash	279
Horenstein, E. A.	189	Virgin, H. I.	389, 630, 954
Hultgren, B.	493	Wai, N.	71
Hylmö, B.	433	Wareing, P. F.	300
Ichioaka, P. S.	538, 552	Wessel, G.	538
Ingestad, T.	336	Wikén, T.	116
Jørgensen, E. G.	840, 846	Woolley, J. T.	538
Karlsson, B.	561	Younis, A. F.	223, 230
Kihlman, B.	493	Yusef, H. M.	852
Király, Z.	877		



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Tentative Method of Sugar Estimation in Laminae and its Application to *Hevea brasiliensis*

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(Received June 3, 1954)

A rapid and reliable method of sugar estimation in laminae, capable of detecting changes in composition which might occur as a result of varied mineral nutrient status, was required. It was thought that such changes might influence the rubber content of the tissue of *Hevea brasiliensis*. For a time the Somogyi method (1945 a) was used in this laboratory but suffered the following disadvantages: (a) non-specificity; thus it could not differentiate glucose from fructose, (b) the clarified leaf extract had to be perfectly colourless before the addition of various reagents and the subsequent colorimetric estimation, (c) the possibility that reducing substances other than sugars might influence the values obtained.

The single dimensional chromatographic method described below enabled an estimate of each individual sugar present to be obtained; it compared favourably with Somogyi's procedure for the estimation of total reducing sugars but gave smaller values for the sucrose, possibly because of (c) above.

The method has been applied to a study of the variation of sugar contents of laminae of *Hevea* grown under different nutrient regimes. It was necessary however before the method was put into routine usage to identify all sugars present in the leaf extracts by means of conventional two dimensional paper chromatographic techniques.

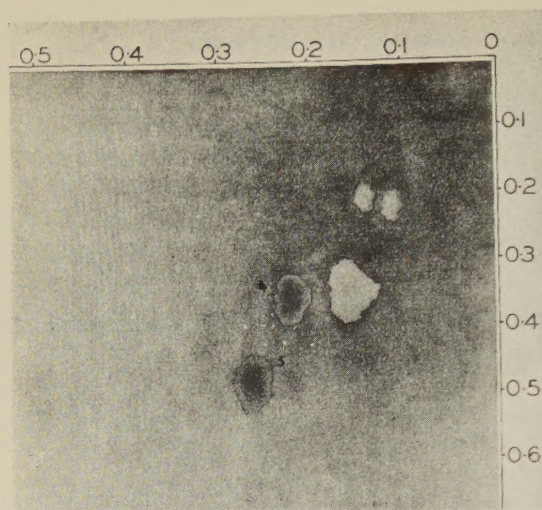


Figure 1. *Two dimensional paper chromatogram of Hevea lamina extract.* Solvents: phenol-water (horizontal R_F scale); butanol-ethanol-water (vertical R_F scale). Dipping reagent: AgNO_3 . Spot 1: meso-inositol, Spot 2: laevo-inositol, Spot 3: sucrose, Spot 4: glucose, Spot 5: fructose.

Method

Preparation of lamina extracts

2 g. of fresh laminae, from which the midribs had been excised, were soxhlet-extracted with 120 ml. of 80 per cent ethyl alcohol for two hours (Laidlaw and Reid 1952). The extract was evaporated under reduced pressure at 30–40° C to approximately 10 ml. This volume was clarified by the addition of 10 ml. of 0.3 N barium hydroxide and 10 ml. of 5 per cent zinc sulphate solution (Somogyi 1945 b), filtered and made up to a volume of 50 ml. with water.

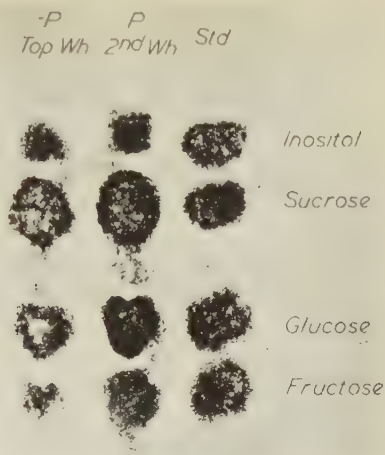
25 ml. of this solution were concentrated under reduced pressure to a volume of 1 ml. from which aliquots, usually 0.01 ml., were taken for the chromatographic estimation of the sugars present.

Identification of sugars present in laminae of Hevea

The identity of the sugars present in clarified alcoholic extracts was investigated by two dimensional paper chromatographic techniques. Butanol-ethanol-water (Partridge 1946) and phenol-water (Partridge 1948) were employed as the solvents and aniline phthallate in butanol (Partridge 1949) and naphthoresorcinol in an alcoholic hydrochloric-phosphoric acid mixture (Bryson and Mitchell 1951) as the dipping agents.

Glucose, fructose and sucrose were always present in the leaf extracts examined. In addition, relatively large amounts of inositol, both meso- and laevo-, occurred. It was possible to distinguish all these compounds on the same paper (Figure 1) by using a silver nitrate dipping agent (Trevelyan, Proctor and Harrison, 1950).

Figure 2. Comparison of standard mixture (20 μ g each of inositol, glucose, fructose; 40 μ g sucrose) with extracts prepared from top (or first) and second whorl laminae of phosphorus deficient plants. Plate obtained by resting chromatogram on photographic paper, exposing to light and printing from negative.



Estimation of sugars present

It was found, using a butanol-ethanol-water solvent, that if a standard mixture of inositol, glucose, fructose and sucrose was developed for 24 hours as a one dimensional descending paper chromatogram, the inositol spot merged with sucrose and the glucose spot with fructose; for a 36 hour run part of the glucose spot merged with fructose. A 48 hour period of development did achieve a separation of each substance; advantage was taken of this, and of the fact that the four substances present could be located by one dipping reagent (silver nitrate), to devise a method for sugar and inositol estimation in lamina extracts.

The method consisted in running one dimensional, descending chromatograms of the alcoholic lamina extracts in a butanol-ethanol-water solvent, 4 : 1 : 5, (Part-ridge 1946) for 48 hours. The papers were dried at 40° C and then dipped in silver nitrate solution (see below). The spots were sufficiently separated to distinguish the three sugars and inositol which were present (Figure 2). When the papers were air-dried the spots were delineated with pencil, cut out, oven dried and weighed. The amount of sugar present in each spot was obtained by reference to a standard curve (relating weight of spot to micrograms of sugar) prepared by spotting on different known amounts of sugar mixtures, and running under the same conditions as the unknowns. 10 to 40 μ g. for each sugar and inositol represented the working range employed (Figure 3). The calibration curves were not exactly linear or reproducible; consequently fresh curves were prepared for each batch of estimations.

Whatman No. 1 filter paper (18 $\frac{1}{4}$ ins. \times 22 $\frac{1}{2}$ ins.) was used throughout the course of this work. The starting line was drawn 7 cms. from the edge of the paper and the concentrated extracts or sugar standards (usually 0.01 ml.), were applied with an Agla syringe, 4 cms. apart. The lower edge of each sheet was regularly serrated to promote even running of the solvent. A "chromatocab" cabinet (supplied by the Research Equipment Corporation, U.S.A.) was used; no temperature control was necessary in this well insulated cabinet. Any temperature variations would pre-

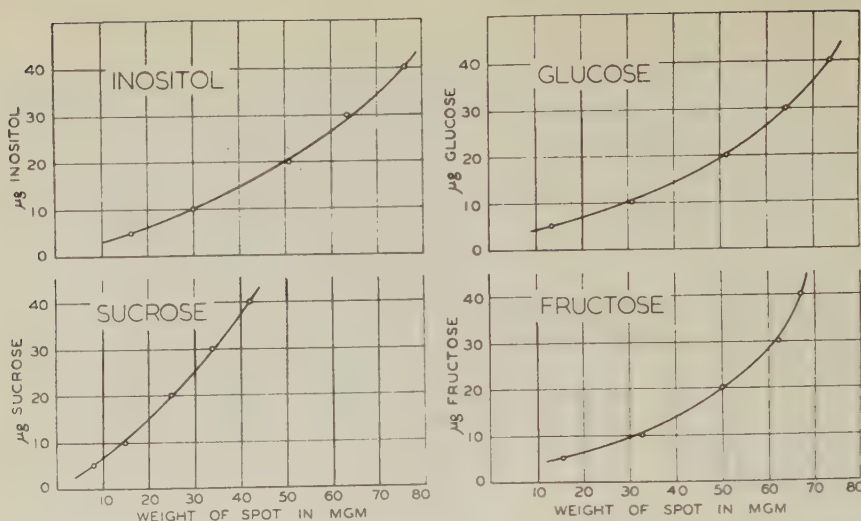


Figure 3. Standard curves obtained by plotting micrograms sugar spotted, against weight of cut out spot (in mg.) using AgNO_3 as the dipping agent.

sumably affect both standards and unknowns similarly; usually the temperature varied between 30—32° C. All solvent troughs, anti-siphoning and anchor rods used, were made of glass.

For the routine estimation of sugar contents of laminae, seven or eight extracts were usually applied to one sheet of paper together with one standard or reference solution containing known amounts of glucose, fructose, sucrose and inositol. Five standard solutions to be used in the preparation of the calibration curves were applied to another sheet in the same cabinet; three out of five of these standards were run in duplicate. Using this procedure thirty to forty lamina extracts were chromatographed at a time.

After the completion of a 48 hour period of development the papers, dried at 40° C, were dipped into a solution of silver nitrate in acetone, air dried, and then further dipped into alcoholic soda as described by Trevelyan *et alii* (1950). After air drying the dipped sheets the sugarspots appeared rapidly and the pencil marking was carried out approximately $\frac{1}{2}$ —1 hour after dipping. It was noted however that the paper could stand for as long as two days without prejudicing the results and occasionally giving less steep calibration curves.

Application of the Method to *Hevea Brasiliensis*

Hevea seedlings of clone Tjirandji 1 were grown in sand culture, using techniques previously described (Bolle-Jones 1954) under conditions of varied mineral status. The treatments were applied by withholding from the plant or reducing its supplies of major nutrients (N, S, P, K, Mg, Ca, Fe) and also

Table 1. *Inositol and sugar contents and some ratio values found in the laminae of Hevea brasiliensis grown under different nutrient regimes. Contents expressed as mg./g. dry lamina.*

Treatment	Inositol		Sucrose		Glucose		Fructose		Sucrose/Glucose		Sucrose/Fructose	
	Whorl		Whorl		Whorl		Whorl		Whorl		Whorl	
	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd
Complete	3.74	4.07	12.02	12.62	2.61	2.09	2.61	3.04	5.23	7.82	4.58	4.2
— N	6.21	2.50	15.92	9.82	3.62	2.55	3.14	3.23	4.60	5.27	5.08	3.2
— S	3.95	2.25	8.67	9.14	1.39	1.29	2.22	2.38	6.72	7.21	3.90	3.9
— P	1.64	2.91	11.49	13.10	2.53	4.09	3.83	4.41	6.20	5.13	3.12	2.9
— K	5.52	2.21	13.73	9.15	4.81	3.56	3.32	1.91	2.94	2.66*	4.15	4.8
— Mg	8.53	2.72	14.26	13.65	1.62	1.59	3.24	3.76	8.80*	8.64	4.40	3.6
— Ca	4.19	2.64	9.00	8.21*	1.94	1.01	2.44	1.70	4.75	8.17	3.73	5.4
— Fe	2.15	3.16	8.98	9.77	1.34	2.02	1.34	2.25	6.70	5.38	6.70*	4.3
— Mn	2.48	2.84	8.59	10.39	2.31	1.89	3.12	3.20	3.75	5.46	2.81*	3.2
— B	4.01	1.71*	14.02	9.45	3.16	1.59	3.12	2.44	4.48	6.02	4.69	4.07
Standard Error	± 1.67	± 0.59	± 1.64	± 1.24	± 0.76	± 0.91	± 0.50	± 0.47	± 1.08	± 1.42	± 0.49	± 0.69

An asterisk * indicates that the treatment differed significantly from the complete nutrient at the 5 % level.

of boron and manganese. The seeds were sown in March 1953 and the laminae, whose sugar contents are given below, were sampled in the following January. At the time of sampling all the deficiency treatments, except boron, produced plants which showed a marked reduction in total dry weight yield.

Leaves from which laminae were taken were fully expanded and usually orientated in the horizontal plane; they were taken from well defined whorl positions. For the sake of convenience the topmost whorl sampled is designated as the first whorl and the successive lower one as the second whorl. Young, limp, pendent leaves were not analysed. Results for the analysis of the first and second whorls are given in Table 1.

The results showed clearly that sucrose was the predominant sugar present in the laminae of *Hevea*. Glucose and fructose, in roughly equal amounts, were present in much smaller quantities, as was inositol.

Lack of magnesium increased the inositol content of the top whorl laminae whereas lack of boron produced a marked decrease in the second whorl laminae.

Nitrogen deficiency caused an accumulation of sucrose in the first whorl laminae but deficiencies of sulphur, calcium, iron and manganese produced the opposite effect. Calcium deficiency also reduced the sucrose content of the second whorl laminae.

Lack of potassium increased the glucose content of the top whorl laminae; lack of phosphorus increased the glucose and fructose contents of the second whorl laminae.

Magnesium deficiency markedly increased the ratio of sucrose to glucose found in the top whorl laminae; lack of potassium decreased this ratio in both top and second whorl laminae.

Restricted iron supply increased the ratio of sucrose to fructose found in the top whorl laminae whereas lack of manganese decreased it.

Discussion

The results presented in Table 1 are given to illustrate the applicability of the method. Some of the effects described did not achieve significance due to the relatively high standard errors obtained; these were ascribed to the limited replication (two) available in the experiment and the lack of uniformity between rubber plants sampled from duplicate plots.

When standard amounts of the sugars or inositol were run in duplicate on three different sheets of paper and the standard errors calculated for the weight of the spots obtained, it was found that the error for a single determination did not vary significantly between the 10, 20 and 30 μg amounts applied. A pooled estimate of the standard error for a single determination for this range (10–30 μg) gave the following values (in mgms.): — inositol ± 2.8 , sucrose ± 2.5 , glucose ± 1.7 , fructose ± 3.5 .

Recovery values for 20 μg amounts of sugar or inositol added to lamina extracts always ranged between 100 and 130 per cent.

The method has the advantages of simplicity, specificity; a large number of samples can be analysed simultaneously. A particular asset of the method is that it enabled the sucrose/glucose and sucrose/fructose ratios to be calculated and their variation with treatment examined. The method can only be applied when the identity of the sugars present in the lamina extract has been definitely established by two dimensional chromatographic procedures. It was found unnecessary to adopt deionisation procedures in studies on *Hevea* lamina extracts.

The method is considered suitable for other botanists who are interested in obtaining a general picture of the effects of various treatments on the concentration and distribution of sugars within the plant, and who do not have the time nor the facilities at their disposal for the operation of complicated but probably more accurate elution procedures.

Summary

The preparation of sugar-containing extracts from the laminae of *Hevea brasiliensis* is described and a one dimensional descending chromatographic method of estimating the individual sugars is given. The chromatograms are

developed for 48 hours, dipped in silver nitrate, and the spots cut out and weighed; the amount of sugar or inositol present in each spot is obtained by reference to a calibration curve prepared from standard amounts of sugars developed under similar conditions. The method is simple and specific and has been applied to the preliminary investigation of the effect of varied mineral nutrient status on the sugar content of the laminae of *Hevea*; these results are described briefly.

The author is indebted to Mr. S. W. Wong for his assistance throughout the course of this work.

References

- Bolle-Jones, E. W.: Nutrition of *Hevea*. 1. Experimental methods. — *J. Rubb. Res. Inst. Malaya* 14: 183. 1954.
- Bryson, J. L. & Mitchell, T. J.: Improved spraying reagents for the detection of sugars on paper chromatograms. — *Nature*, (Lond.) 167: 864. 1951.
- Laidlaw, R. A. & Reid, S. G.: Analytical studies on the carbohydrates of grasses and covers. 1. Development of methods for the estimation of the free sugar and fructosan contents. — *J. Sci. Food Agric.* 3: 19. 1952.
- Partridge, S. M.: Application of the paper partition chromatogram to the qualitative analysis of reducing sugars. — *Nature* (Lond.), 158: 270. 1946.
- Filter paper partition chromatography of sugars. 1. General description and application to the qualitative analysis of sugars in apple juice, egg white and foetal blood of sheep. — *Biochem. J.* 42: 238. 1948.
- Aniline hydrogen phthalate as a spraying agent for chromatography of sugars. — *Nature* (Lond.), 164: 443. 1946.
- Somogyi, M.: A new reagent for the determination of sugars. — *J. biol. Chem.* 160: 61. 1945 a.
- Determination of blood sugar. — *J. biol. Chem.* 160: 69. 1945 b.
- Trevelyan, W. E., Proctor, D. P. & Harrison, J. S.: Detection of sugars on paper chromatograms. — *Nature* (Lond.), 166: 444. 1950.

Studies in Physiological Analysis of Yield. II. Further Observations on Varietal Differences in Photosynthesis in the Leaf, Stem and Ear of Wheat

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Introduction

In the previous paper of this series the writers (Asana and Mani, 1950) furnished evidence for the existence of varietal differences in photosynthesis in the leaf, ear and stem of wheat during the post-ear emergence period. It is proposed to consider critically in this paper all the observations made so far on the subject with a view to determining the effect of season, the causes of varietal differences in photosynthesis in different organs and the relation of photosynthesis during the post-ear emergence period to grain yield. The experimental details and the methods of calculation were described in the earlier publication. It may be recapitulated that apart from the control, the experimental treatments, which commenced 5 days after anthesis, consisted of defoliation, shading of the ear, and a combination of these two. Only mother shoots were utilized in this study and tillers were removed at the time of commencement of treatments and whenever they appeared later. Both initial and final (harvest) plant samples were collected and the changes in the dry matter of the leaf, stem with sheaths, grain and husk (rachis, awns and glumes) were determined. From the mean effects of defoliation and shading of ear on the changes in the dry matter, an estimate was made of the contribution by photosynthesis in the leaf and ear respectively to the increase in the dry weight of the plant (referred to henceforth as net assimilation

Table 1. *Seasonal variation in photosynthesis in leaf ear and stem.*

Season	Contribution in gm. to N.A. by			N.A. in gm.	Significant Differences			
	Leaf (L)	Ear (E)	Stem (S)					
Variety N.P. 710								
1947	0.306	0.786	0.183	1.275	N.A.	1951	1950	1948 1947
1948	0.469	0.726	0.386	1.581	L	1950	1951	1948 1947
1950	1.073	0.648	0.000	1.721	E	1951	1947	1948 1950
1951	0.825	0.842	0.430	2.097	S	1951	1948	1947 1950
	0.785	0.741	0.219					
Variety N.P. 165								
1947	0.459	0.297	0.311	1.067	N.A.	1948	1950	1947
1948	0.573	0.370	0.669	1.612	L	1950	1948	1947
1950	0.837	0.195	0.160	1.192	E	1948	1947	1950
	0.694	0.257	0.307		S	1948	1947	1950
Variety Punjab 9 D								
1947	0.282	0.412	0.279	0.943	N.A.	1948	1951	1947
1948	0.431	0.511	0.785	1.727	L	1951	1948	1947
1951	0.611	0.653	0.446	1.710	E	1951	1948	1947
	0.484	0.557	0.481		S	1948	1951	1947
Variety N.P. 52								
1948	0.599	0.609	0.840	2.048	N.A.	1948	1950	
1950	1.247	0.615	0.053	1.809	L	1950	1948	
	1.061	0.613	0.202		E	1950	1948	
					S	1948	1950	

or N.A.), while the effect of combined treatments gave an estimate of the contribution by photosynthesis in the stem.

Analysis of variance was applied in the statistical examination of the data and differences at 5 per cent level of probability only were considered significant.

Experimental results

The data on the contributions from different organs to net assimilation (N.A.) are given in Table 1.

It may be concluded from the data in Table 1 that the contribution from photosynthesis in the ear and the leaf to net assimilation was, on the whole, larger than that of the stem and that the contribution from the ear showed much less variation with season than that from the leaf and the stem.

Table 2. *Varietal differences in photosynthesis in leaf ear and stem.*

Variety	Contribution to N.A. in gm by			N.A. in gm.	Seasons	Significant Differences
	Leaf (L)	Ear (E)	Stem (S)			
N.P. 165	0.694	0.253	0.253	1.200	3	N.A. N.P. 710 > N.P. 165
N.P. 710	0.768	0.696	0.125	1.589		E " > "
	0.731	0.475	0.189			
Punjab 9 D..	0.484	0.557	0.482	1.523	3	N.A. N.P. 710 > Pb. 9 D
N.P. 710	0.606	0.799	0.357	1.762		E " > "
	0.545	0.678	0.419			L " > "
						S " < "
N.P. 52	1.062	0.613	0.202	1.877	2	None
N.P. 710	0.900	0.671	0.109	1.680		
	0.981	0.642	0.155			
N.P. 165	0.516	0.324	0.368	1.209	2	E N.P. 710 > Others
Punjab 9 D..	0.356	0.461	0.517	1.335		S Pb. 9 D > N.P. 710
N.P. 710	0.387	0.756	0.284	1.428		
Agra Local..	0.504	0.514	0.409	1.427		
	0.446	0.520	0.399			

Comparison of the varieties in respect of contributions from the different organs to net assimilation is made in table 2. It will be seen that varietal differences in photosynthesis in leaf, ear and stem do exist. The causes of these varietal differences may now be examined.

The variation in ear contribution may be due to variation in spikelet number, presence of awns, time of anthesis and thus the period during which the ear remains green and functioning, and variation in photosynthetic rate if any.

The mean spikelet number of the four varieties varied as follows:

N.P. 710	Agra Local	Punjab 9 D	N.P. 165
19.0	18.4	16.5	16.3

It may also be noted that the ear of N.P. 165 is awnless, whereas that of the other three varieties is awned. The higher ear-contribution of N.P. 710, as compared to that of N.P. 165 and Punjab 9 D can thus be accounted for. The smaller ear-contribution in Agra Local as compared to that of N.P. 710 and the equal ear-contribution of N.P. 165 and Punjab 9 D cannot, however, be explained on the basis of variation in spikelet number or presence of awns. The ear of N.P. 165 dehisces about ten days earlier than that of N.P. 710 and about a fortnight earlier than that of Agra Local and Punjab 9 D. It is quite likely that with the usual tendency for atmospheric drought to increase from March onwards, under Delhi conditions, the photosynthetic activity of the ear might decline and thus the ears that dehisce later might be at some disadvantage. Although late dehiscence might perhaps account for the

Table 3.

Mean number of green leaves per shoot (control).

Variety	8/3	14/3	19/3	25/3	29/3	3/4
N.P. 165	3.8	3.4	2.9	2.3	1.3	0.7
N.P. 52	4.0	3.5	3.0	2.1	1.3	0.7
N.P. 710	4.0	3.2	2.5	1.7	0.9	0.4

Rate of yellowing of stem (control).

Variety	Mean length of stem in cm.	Length in cm. of yellow portion on					
		15/3	19/3	25/3	29/3	5/4	10/4
N.P. 165	86.3	1.4	3.5	18.3	32.4	46.3	57.5
N.P. 52	85.2	1.7	6.6	35.5	47.5	64.1	76.1
N.P. 710	79.9	3.4	7.9	29.0	43.5	63.7	69.1

similarity of ear-contribution of the awned varieties, Agra Local and Punjab 9 D, with that of the awnless variety N.P. 165, it cannot explain the lower contribution of the ear of Agra Local as compared to that of N.P. 710. Similarly the similar ear-contribution of Agra Local and Punjab 9 D in spite of variation in spikelet number is difficult to account for. Vervelde (1953) has suggested that the influence of the awns may depend upon their length and thus may account for the variation in their contribution to yield of different cereals. The length of the awns did not, however, materially differ in the three awned varieties.

In the 1951—52 season, observations were recorded on the functionally active period of the ears of N.P. 710 and Punjab 9 D and the mean number of days taken for complete yellowing of the ear was 39 days in N.P. 710 and 42 days in Punjab 9 D.

From the above considerations it would, therefore, appear that there might exist a varietal difference in the photosynthetic rate of the ear.

The varietal difference in leaf-contribution may be due to differences in the size and number of leaves and in their functionally active period (as judged by the rate of yellowing). The data obtained during 1950—51 are presented in Table 3. During this season the contribution from the leaf of N.P. 165 to net assimilation as well as grain yield¹ was significantly lower than that of N.P. 710 and N.P. 52 respectively, the latter two not differing significantly from each other; the contribution from the stem was, however, more or less identical in the three varieties. The rate of yellowing of leaves, was on the whole quicker in N.P. 710 than in N.P. 165 and N.P. 52; the

¹ The calculation of contributions from different organs to grain yield was made according to method referred to in the next chapter.

Table 4.
Mean number of green leaves per shoot (control).

Variety	27/2	3.3	12/3	21.3	25.3	31/3
Kendee	4.7	3.8	3.2	2.7	1.9	0.6
N.P. 710	4.0	3.3	2.7	1.9	1.0	0.2
Punjab 9 D	4.9	3.6	3.1	2.3	1.6	0.4

Variation in length and width of flag leaf.

Mean length in cm.			Mean width in cm.		
N.P. 710 16.9	Punjab 9 D 14.4	Kendee 13.7	N.P. 710 1.50	Kendee 1.19	Punjab 9 D 1.14

yellowing of stem was, on the other hand, quicker in N.P. 52 and N.P. 710. Even in the field this striking difference has been consistently noticed. It is clear that these differences do not explain the varietal differences in leaf- and stem-contribution.

The data obtained during 1951–52 are presented in Table 4.

The mean leaf contribution to 'net assimilation' varied in the order Kendee = N.P. 710 > Punjab 9 D. Assuming that the mean total leaf area of the three varieties varied in the same order as for the flag leaf it appears that the functionally effective leaf area in N.P. 710 was substantially larger than that of Kendee as well as Punjab 9 D upto 21st March; subsequently, however, the effective leaf area of Kendee was more than that of the other two varieties. The higher leaf-contribution in N.P. 710, as compared to that of Punjab 9 D can accordingly be satisfactorily accounted for. In view of the comparatively longer period during which the effective leaf area of N.P. 710 exceeded that of Kendee, the equality of their leaf contributions is rather surprising. Either more critical observations are required or it may be that there is some difference in the photosynthetic activity of the leaves of the two varieties. Watson (1952) also reports a substantial difference in the 'net assimilation rate' of sugarbeet strains. On the other hand, Heath and Gregory (1938) are of opinion that during the vegetative phase the mean net assimilation rate is constant for all species and environments; Chapman and Loomis (1953) also did not find any difference between potato varieties in respect of rate of CO₂ absorption per unit leaf area. The mean leaf contribution to grain yield, however, varied in the order N.P. 710 > Kendee > Punjab 9 D, which tallies very well with the order of effective leaf area.

The rate at which the stem yellowed was estimated by the number of days taken for complete yellowing of the stem. The variation was in the order

Table 5. *Relation between net assimilation and grain yield.*

Variety 1	Season 2	Net Assimilation in gm. 3	Grain yield in gm. 4	Difference between 3 and 4	Mean grain No. per ear 5
N.P. 165	1948	1.61	1.44	+ 0.17	34.9
	1950	1.19	1.78	— 0.59	42.2
N.P. 710	1948	1.58	1.65	— 0.07	41.0
	1950	1.72	2.34	— 0.62	55.0
	1951	2.09	2.16	— 0.07	51.3
N.P. 52	1948	2.05	1.74	+ 0.31	47.1
	1950	1.81	2.26	— 0.45	61.7
Punjab 9 D {	1948	1.73	1.50	+ 0.23	35.7
	1951	1.71	1.60	+ 0.11	41.8

Kendee > N.P. 710 = Punjab 9 D. The stem-contribution to 'net assimilation' also varied in the same order and here the apparent correlation with functionally active period seemed satisfactory. The stem contribution to grain yield was, however, the highest in N.P. 710, it being significantly superior to that of Punjab 9 D only. It will be seen that this order did not correlate satisfactorily with differences in the rate of stem-yellowing.

In table 5 the data relate to controls only and the data of 1947 have not been included because the initial grain weight was not determined during that season. The grain yield in table 5 represents the difference between final grain weight and the initial grain weight i.e. the actual grain weight that accumulated after the commencement of the treatments (five days after anthesis). It may be noted that the initial grain weight constituted about 3 per cent of the final weight.

It can be seen from table 5 that in some cases the grain yield exceeded net assimilation, whereas in others the reverse was the case. There does not appear to be any direct relation between net assimilation and grain yield, whereas between grain number and grain yield there seems to be a good correspondence. This disparity between grain yield and net assimilation requires consideration. Grain weight would exceed net assimilation if there were a decrease in stem weight. Does the decrease in stem weight involve contribution of material to the grains? From extensive experimental evidence Archbold (1945) questions the role of stored sugar in the stem as reserve for the ear of barley and, in fact, regards its decrease as largely due to senescence. She did not also find any appreciable breakdown of polysaccharides. It is not possible directly to test this view from the present data. In Figure 1 is plotted loss in stem weight against grain weight. Under control and ear-shading grain yield increases with increase in stem loss at a greater

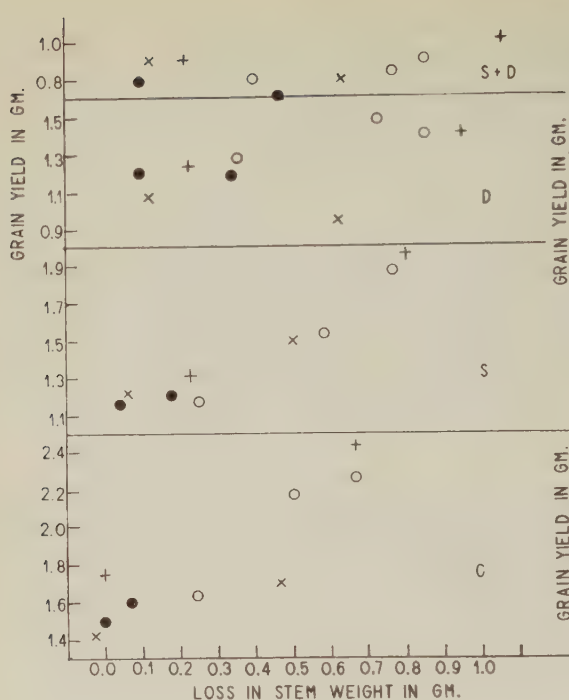


Figure 1.

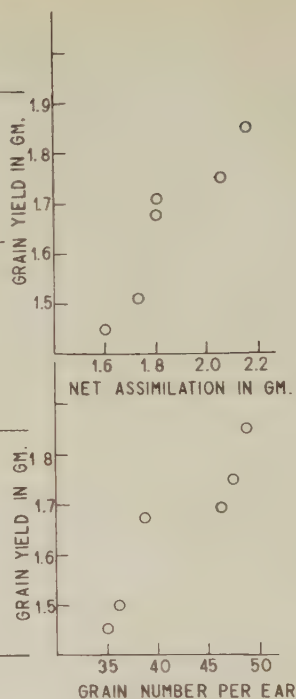


Figure 2.

Figure 1. *The relation between grain yield and loss in stem weight.*

N.P. 710

N.P. 165

Punjab 9 D

N.P. 52

Figure 2. *The relation between grain yield, number of grains and net assimilation rate.*

rate than under the other two treatments. Under the two defoliation treatments, respiration might have increased due to wounding thus involving increased loss of sugar and as such the relation between decrease in weight of stem and grain yield might be obscured. It is, however, realised that the relationship between decrease in stem weight and yield cannot be regarded as direct evidence in favour of supply of reserve material from the stem to the ear.

Another argument advanced by Archbold against the migration theory is that under treatments such as defoliation, ear-shading etc. the loss of stem sugar might be expected to be larger, since such treatments are expected to put part of the photosynthetic system out of action and should consequently involve enhanced migration of reserve material from the stem. She did not

find any evidence of increased loss of sugar or polysaccharides from the stem under such treatments. From the data in table 7 it appears that defoliation, in six cases out of nine, involved increase in loss of stem weight and ear-shading in three out of nine. Increase in loss of weight of stem due to defoliation, may perhaps be attributed to increased respiration due to wounding, but no such simple explanation of the effect of ear shading can be offered. In conclusion, the writers feel inclined to revise the view, accepted in the earlier paper, about transfer of reserve material from the stem to the ear and to regard the question as open considering the insufficiency of experimental evidence. As such the calculation of contribution of reserves from stem to grain weight, made in the earlier paper, cannot be justified. Again the calculation of treatment effects on husk-weight, made in the earlier paper, is open to objection in view of the fact, revealed from the data of several seasons, that the husk weight increased under control and defoliation only (control > defoliation), whereas under the other two treatments, it remained more or less constant or decreased sometimes.

The method of estimating the contribution from photosynthesis in different organs to net assimilation (increase in plant weight) alone seems to be justified. That this estimate is also not completely above reproach is indicated by the distinctive effect of defoliation on loss in stem-weight. Due to the greater effect of defoliation on loss in stem-weight the value of net assimilation under this treatment (as well as defoliation plus ear shading) is underrated and since the contribution from different organs is estimated from the reduction in total assimilation, the effect of defoliation as well as defoliation plus ear shading would be exaggerated. In a comparative study of varieties, however, this defect in the estimate of leaf-contribution may not perhaps be regarded as serious.

It must be admitted that the experimental technique adopted here is not efficient enough for a correct estimate of the photosynthetic contribution from different organs to grain yield. The drawbacks of this technique have also been pointed out by Porter and her associates (Porter et al 1950). Direct estimates are, however, almost impossible of practical achievement with a large number of varieties involving a continuous record of photosynthetic activity. The chief obstacle to equating net assimilation to grain yield has been the decrease in weight of stem. In six cases, where no such decrease was noticed, the grain yield was plotted against net assimilation as well as grain number and the relation was found to be equally satisfactory in both the cases (Figure 2). In view of this relationship and of the fact that the maximum disparity between grain yield and net assimilation was of the order of 25 per cent it is perhaps reasonable to suggest that grains are filled up almost entirely by the current assimilation. Boonstra (1936) showed that

Table 6.

Variety		Contribution to grain by			
		Leaf	Ear	Stem	Grain weight in gm.
N.P. 710	{Actual	0.65	0.52	0.85	2.02
	{Percentage ...	32 %	26 %	42 %	
N.P. 165	{Actual	0.54	0.18	0.84	1.56
	{Percentage ...	36 %	11 %	53 %	
Punjab 9 D	{Actual	0.35	0.39	0.81	1.55
	{Percentage ...	22 %	26 %	52 %	
N.P. 52	{Actual	0.79	0.48	0.97	2.24
	{Percentage ...	36 %	21 %	43 %	

in wheat the grain was filled by carbohydrates synthesized during the period of its development and not by those of the nature of reserves and that only those organs, which were capable of photosynthesis during about a 5-week period of grain development, contributed directly to the grain. Porter, Pal and Martin (1950) also arrived at a similar conclusion in the case of development of the barley grain. Assuming that all the increase in grain weight was due to current assimilation and almost independent of supply of reserve material from the stem the mean contribution from different organs to grain yield is indicated in table 6.

It will be seen that in the three varieties with bearded ears viz. N.P. 710, N.P. 52 and Punjab 9 D, contribution from the ear is substantially larger than that in N.P. 165 and among the three bearded varieties the contribution of the ear of Punjab 9 D is the smallest. It may be noted that the extent of contribution by ear to grain weight is more or less the same as that found by Porter, Pal and Martin (1950) and Watson and Norman (1939) for the ear of barley, and by Boonstra (1936) for the ear of wheat. These estimates are more or less in agreement with those derived earlier in the case of net assimilation. Similarly the contributions from leaf to grain weight also vary with the varieties in more or less the same order as indicated earlier in the case of net assimilation. The extent of contribution by the stem to grain weight, is however, much greater than that towards net assimilation.

Table 7. Comparison of losses in stem-weight under control (C), defoliation (D), ear-shading (S) and defoliation plus ear-shading (D+S).

Seasons	N.P. 710	N.P. 165	Punjab 9 D	N.P. 52
1948.....	<u>D+S</u> <u>D</u> <u>C</u> <u>S</u>	<u>D+S</u> <u>D</u> <u>S</u> <u>C</u>	<u>D+S</u> <u>D</u> <u>S</u> <u>C</u>	<u>D</u> <u>S</u> <u>D+S</u> <u>C</u>
1950.....	<u>D+S</u> <u>D</u> <u>S</u> <u>C</u>	<u>D</u> <u>D+S</u> <u>S</u> <u>C</u>	—	—
1951.....	<u>D+S</u> <u>D</u> <u>S</u> <u>C</u>	—	<u>D+S</u> <u>D</u> <u>S</u> <u>C</u>	<u>D+S</u> <u>D</u> <u>S</u> <u>C</u>

Discussion

Since the grain appears to be filled almost entirely by the material photosynthesized after anthesis, a detailed study of the organs carrying on photosynthesis during the post-ear emergence period could be expected to point to suitable indices of yield. Boonstra (1929) found that four varieties of oats differed considerably in the length of life of corresponding leaves and that high yield was associated with long life of the leaves. Boonstra (1937) also accounted for the high yield of strain A and the high-sugar content of strain Z of sugarbeet variety Kuhn on the basis of leaf area and longevity. Watson (1952) has shown the usefulness of determining leaf area index in relation to yield. Our observations point to the existence of varietal differences in the contributions made by different organs to grain yield. Although observations on the rate of yellowing of the three organs, their size, presence of awns, etc. did not completely account for these varietal differences, it may be suggested that further refinement in these observations would perhaps improve the correlation.

Boonstra (1929) showed that in wheat only the top internode and peduncle, with their attached leaves, were effective in supplying material to the ear. Archbold (1942) found that while removal of leaves only reduced the dry weight of the ear by 10 per cent or less, additional removal of the flag-leaf sheath reduced the ear dry weight by 28 per cent. These observations may have a practical significance. Under field conditions the ear, the peduncle and the flag-leaf would be exposed to the maximum light-intensity and their full potentiality for photosynthetic activity would thus find expression. It would, therefore, be worthwhile to compare the physiological behaviour of the flag-leaf with its sheath and the peduncle of different varieties.

Tillers were omitted in this study for the sake of simplifying the experimental technique. It is however, essential to carry out these observations on whole clumps over a number of seasons and under varying conditions of nutrient and water supply. If the contribution from even one of the three organs were influenced more strongly by environment than by heredity, attempts to improve yield by breeding for these attributes may not meet with much success.

Summary

From experiments involving defoliation, shading of ear and defoliation plus ear-shading of mother-shoots, 5 days after anthesis, over a number of seasons, more confirmatory evidence was obtained for the existence of varietal differences in the contribution from photosynthesis in the leaf, ear

and stem of wheat to increase in plant weight (net assimilation) during the post-ear emergence period.

The seasonal variation in the contribution from the ear to net assimilation was much smaller than that from the foliage and stem. The varietal difference in the ear-contribution with a few exceptions, appeared to be related to the presence of awns and difference in spikelet number. The varietal differences in the contribution from the leaves and stem to net assimilation could be accounted for to some extent on the basis of their functional area, as judged by persistence of green colour.

The grain yield was not always found to be identical with net assimilation, the apparent reason being the decrease in stem weight after ear emergence. The loss in weight of stem was much larger due to defoliation and only occasionally larger under ear-shading as compared to control. The increase in loss of stem-weight, under control and ear-shading, is not regarded as an indication that reserve material from the stem contributed substantially to grain because the loss in stem-weight did not consistently increase due to ear-shading, as may be expected when carbohydrate is in short supply. Assuming on this evidence that the reserve material from the stem contributed but little to the grain and considering the identity of grain weight with net assimilation in a few cases, where stem weight did not decrease after anthesis, it is suggested that the grain is filled almost entirely by the material photosynthesized after anthesis. As such, the effects of treatments on grain yield were estimated and more or less the same order of varietal differences in the contributions from different organs was obtained as in the case of net assimilation. It is, therefore, suggested that varietal differences in yield may perhaps be accounted for by such attributes as leaf and stem size, spikelet number and awns of ear and their functional period and that a detailed study along these lines on whole clumps and under varying environmental conditions may point to suitable indices of yield.

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References

- Archbold, H. K.: Physiological Studies in Plant Nutrition. XIII. Experiments with Barley on Defoliation and Shading of Ear in Relation to Sugar Metabolism. — *Ann. Bot.* N.S. 6: 487. 1942.
- Some Factors concerned in the Process of Starch Storage in the Barley Grain. — *Nature* (London) 156: 70. 1945.
- Asana, R. D. & Mani, V. S.: Studies in physiological Analysis of Yield I. Varietal Differences in Photosynthesis in the Leaf, Stem and Ear of Wheat. — *Physiol. Plant.* 3: 22. 1950.

- Boonstra, A. E. H. R.: Quoted by Watson (1952). — 1929.
— Quoted by Vervelde (1953). — 1936.
— Quoted by Watson, D. J. (1952). — 1937.
- Chapman, H. W. & Loomis, W. E.: Photosynthesis in the Potato under Field Conditions. — *Plant Physiol.* 28: 703. 1953.
- Heath, O. V. S. & Gregory, F. C.: The Constancy of the mean Net Assimilation Rate and its ecological Importance. — *Ann. Bot. N.S.* 2: 811. 1938.
- Porter, H. K., Pal, N. & Martin, R. V.: Physiological Studies in Plant Nutrition. XV. Assimilation of Carbon by the Ear of Barley and its Relation to the Accumulation of dry Matter in the Grain. — *Ibid.* 14: 55. 1950.
- Vervelde, G. J.: The agricultural Value of Awns in Cereals. — *Netherl. J. Agri. Sci.* 1: 2. 1952.
- Watson, D. J. & Norman, A. G.: Photosynthesis in the Ear of Barley and Movement of Nitrogen into the Ear. — *J. Agr. Sci.* 29: 321. 1939.
- Watson, D. J.: The physiological Basis of Variation in Yield. — *Adv. Agron.* 4: 101. 1952.

The Biochemistry of Lignin Formation

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Hydroxylated phenylpropanes are believed to be the principal structural components of lignins (Freudenberg, 1939) but investigations have only recently begun to establish a concrete picture of the chemical processes in lignification.

Freudenberg *et al.* (1952, 1953) prepared cell-free extracts from mushroom and green plant tissues and with these converted coniferyl alcohol and hydroxycinnamyl alcohols into dehydrogenation polymers of a lignin-like character. Evidence was presented for the participation of peroxidase in the reaction. Freudenberg and Bittner (1953) demonstrated subsequently that the radioactivity of C¹⁴-labeled coniferin sealed under the bark of a spruce could be recovered almost entirely in the lignin fraction of the wood.

Freudenberg's slow polymerization of phenylpropanes, several days to weeks in duration, was hastened to a reaction requiring only a few minutes to several hours by Siegel (1953, 1954) who used tissue slices instead of isolated oxidizing enzyme preparations. Tissues derived from several vascular plants transformed eugenol and other hydroxyphenyl propanes into lignins localized in the cell wall by means of H₂O₂-dependent reactions. The parallel tissue distribution patterns of peroxidase, endogenous lignin, and lignin-forming capacity, as well as peroxide-dependency showed that the conversion of eugenol and related substances to lignin-like polymers was mediated by a peroxidative step. Further, the potato tuber transformed eugenol to

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lignin only in the presence of peroxide, its high phenolase content notwithstanding.

The capacity of peroxidase to convert suitable substrates into lignin marks this enzyme as a significant one in the differentiation of the vascular plant. Van Fleet (1947) concluded from histochemical studies that peroxidase appeared to be correlated with differentiation, however, he could not, at that time, specify the manner in which the two were related.

Initial observations on formation of lignin from eugenol were based on color tests, isolation with 72 per cent sulfuric acid, and general solubility of the product, but these data were augmented subsequently by more detailed examination of the product including elementary and group analysis. The findings altogether suggest that the peroxidation of eugenol *in vivo* yields a product similar in its characteristics to coniferous lignins.

Some consideration was also given to the genesis of peroxide in the plant; propionaldehyde was found to increase the oxidation of pyrogallol by bean tissue (Siegel, 1954). Siegel and Galston (1954) demonstrated that flavo-protein substrates, MnCl_2 , 2,4-dichlorophenol and light enhance peroxide formation by pea tissue and extended the work of Andreae and Andreae (1953) showing indoleacetic acid to be a peroxide-generating substance.

The present work explores further some biochemical aspects of the lignification process extending those lines of investigation previously begun.

Chemical Properties of Eugenol Lignin Formed in Pea Roots

Terminal 5 mm portions of 3-4 day old Alaska pea roots were excised and 1-2 gm. samples (fresh wt.) incubated for 10-15 hrs. in 25 ml. of .05 M KH_2PO_4 containing 0.3-0.4 mmol. of eugenol and 0.5-1.0 mmol. of H_2O_2 . The tissues were then extracted once with 25 ml. boiling water, three times with boiling ethanol and once with boiling chloroform, a procedure adopted routinely in previous work (Siegel, 1953). Extractants were removed and tissues placed in 72 per cent sulfuric acid for 15 hrs. at 2°C. After digestion, the mixture was diluted to 3 per cent acid and autoclaved (15 pounds pressure, 120°C.) for 3 hrs. The carbohydrate-free residue was then washed free of acid by centrifugation and dried.

Isolated eugenol lignin contained a dioxane-soluble fraction which constituted 40-60 per cent of the total dry weight. For comparison, the lignins of oak, spruce and pine were soluble in dioxane to the extent of 20-30 per cent of their weight. Lignin formed in isolated cell walls (preparation to be described below) and lignin isolated from excised vascular bundles of a fern (*Woodwardia* sp.) were soluble in dioxane to the extent of 20 and 10 per cent respectively. Although none of these lignins could be dissolved directly in ethanol to more than a slight degree, their dioxane fractions could

be dried and redissolved easily in the alcohol. Such an increase in the ethanol solubility after dioxane treatment suggests that the latter solvent modified the lignins chemically, possibly as a consequence of traces of impurities (Brauns, 1950).

The dioxane-soluble fraction of eugenol lignin was concentrated to dryness and the solids redissolved in 95 per cent ethanol. Five volumes of water were then added to the deep yellow alcoholic solution, precipitating the lignin which was then recovered as a light brown product by centrifugation at $10,000 \times g$. Solution and precipitation from ethanol were then repeated twice. Both the phloroglucinol-HCl and chlorine-sulfite color tests were given by this partially purified lignin.

Pea root tissue was incubated with 0.375 mmol. of eugenol with or without 0.5 mmol. of H_2O_2 in 25 ml. of buffer for 10 hrs. at 24°C. To determine the active cellular fraction, comparable groups were prepared containing: (a) 1 gm. fresh wt. of 2 mm. tissue slices, (b) the washed cell wall material from 1 gm. of tissue, and (c) the wall-free brei from 1 gm. of tissue. Cell wall material was prepared by grinding frozen tissue in a mortar chilled to $-20^\circ C$. suspending in 10 ml. of .05 M KH_2PO_4 , centrifuging at $3,000 \times g$. The entire process was repeated twice more before wall fragments were used. After incubation, groups a and b were processed as usual for lignin; group c was centrifuged at $10,000 \times g$ to sediment any lignin which might have been formed.

All groups yielded substances positive by the phloroglucinol-HCl and chlorine-sulfite color tests, but the wall-free brei yielded only traces embedded in less than 1 mg. of protein. For other groups the rates of lignin deposition were: sections, 3.46 mg./100 mg. dry matter/hr.; cell wall, 2.68 mg./100 mg. dry matter/hr.

Table 1. *Properties of Some Lignins and the Lignin-forming System in Peas.*

Property	Natural Lignins			Eugenol	Eugenol-lignin formed by	
	Oak	Spruce	Pine		Tissue Slices	Cell Walls
Composition %						
C	58-60	65-66	62-67	73	63	58
H	5	6	5-6	7	7	6
OCH ₃	20-22	15-16	14-16	19	15	10
Solubility						
Water	—	—	—	+	—	—
KOH	+	+	+	+	+	+
Ethanol	—	—	—	+	—	—
Acetone	—	—	—	+	—	—
Dioxane	+	+	+	+	+	+
UV Spectrum						
Max. m μ [†]	275	281	278	282	280	280
Min. m μ	265	270	267	252	270	276
Color with Cl ₂ + Na ₂ SO ₃	Red	Red	Red	Yellow	Red	Red

[†] All substances have additional maxima below 230 m μ .

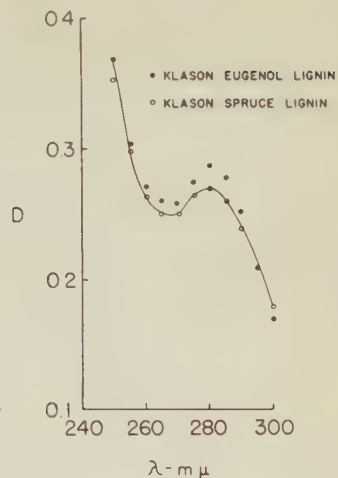


Figure 1. The ultraviolet absorption spectra of dioxane-soluble fractions of spruce and eugenol (synthetic) lignins.

The chemical properties of eugenol, synthetic lignins, and natural lignins are compared in table 1. Eugenol may be distinguished from both natural and synthetic lignins. In common with the lignins, eugenol has an absorption maximum between 275 and 285 $m\mu$, but the preceding minimum is quite distinctive. The ratio, density at 280 $m\mu$ /density at preceding minimum is 1.07—1.2 for all of the lignins studied but 4.5 for eugenol. Absorption spectra were determined with the Beckman model DU spectrophotometer on 95 per cent ethanol solutions: dioxane extracts redissolved in the alcohol were used.

Isolated, washed cell wall material retained 3—6 percent of the total peroxidase activity of the whole tissue. Cell wall lignin is richer in oxygen and its methoxyl content is at the lower limit for known lignins, possibly as a consequence of different concentrations of reactants, more rapid diffusion, and differences in homogeneity of the whole system.

The similar spectral characteristics of natural and synthetic lignins (table 1) have been detailed for spruce and eugenol Klason (72 percent H_2SO_4) lignins (figure 1). The values found for eugenol lignin follow closely the curve for the natural substance but show the two materials to differ in some degree. Synthetic lignin was formed from a pure substance with at most negligible contribution of endogenous phenols whereas the natural lignification process may involve polymerization of several phenylpropanes, and in consequence of secondary, non-enzymatic oxidations, other phenols as well.

A sample of eugenol lignin was degraded by oxidation with alkaline nitrobenzene. Following essentially the procedure of Freudenberg *et al.* (1940)

vanillin was formed and detected with *m*-aminobenzenesulfonic acid. The yield of vanillin was not determined, but natural lignins yield as much as 25 percent of the aldehyde (Brauns, 1952).

Kinetics and Stoichiometry of Lignin Formation

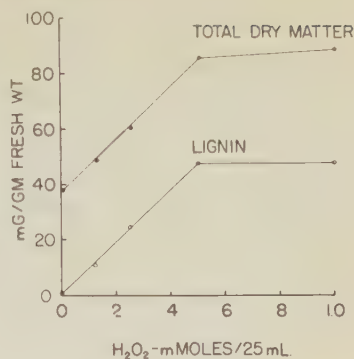
Visually observable changes which occur during lignin formation suggest the sequence of events in oxidation of eugenol. These observations were made on a variety of reaction mixtures containing 0.25–0.5 mmol. of eugenol and pea root sections in 25 ml. of .05 *M* KH_2PO_4 . Within 30 seconds after adding 0.25–1.0 mmol. of H_2O_2 to reaction mixtures, the ambient medium becomes turbid and develops a deep yellow color which increases in intensity for 5–30 minutes. After full development of color in the medium, the tissue previously white or slightly yellow, takes up the ambient yellow pigment leaving the solution almost devoid of color. The intensely yellow tissues now develop a progressively deeper brown coloration over a variable period. The usual color reactions for lignin are first given by an acid and alcohol insoluble product concurrently with browning of the yellow pigment. The yellow substance is readily reduced to a colorless form with ascorbic acid or cysteine, thus may be a reversibly oxidized intermediate between eugenol and its polymeric derivatives. Certain peroxidase substrates, benzidine for example, form a series of colored substances when reacted with H_2O_2 -peroxidase. Such color sequences, often observed in the oxidation of hydroxy- and aminobenzenes, indicate compulsory univalent electron transfer associated with semi quinone formation (Michaelis, 1935). It may also be pointed out that aqueous solutions of eugenol are colorless in acid solution and intensely yellow above pH 8. The lack of anticipated optical activity in natural lignins and their sulfonic acids led Adler and Lindgren (1952) to suggest the participation of radicals in the polymerization of aromatic substances to lignin.

The peroxide-dependency of the lignin-forming system has been demonstrated, however quantitative relationships have not been detailed previously.

One gram (fresh weight) samples of pea root sections were placed in 25 ml. of .05 buffer containing 0.38 mmol. of eugenol and varying amounts of peroxide. After 15 hrs. incubation with continuous shaking, tissues were dried to constant weight at 100° C., and the lignin content determined as usual.

The increase in total dry matter with increasing H_2O_2 parallels almost exactly the variation in lignin synthesized (figure 2). Addition of more than 0.5 mmol. of peroxide caused no further alteration in lignin produced from the quantity of eugenol supplied. From 62 mg. of eugenol, 43 mg. of lignin

Figure 2. The relation of H_2O_2 concentration to synthesis of eugenol lignin in pea sections and to increase in dry matter. (0.38 μ Moles of eugenol supplied; mixture incubated 15 hrs. at 24° C.).



were formed; the lignin-synthesizing system appears to be highly efficient. The total contribution of peroxide to the lignin produced cannot have exceeded 8 mg., the amount of active oxygen in 0.5 mmol. of H_2O_2 . Available peroxide oxygen was presumably reduced in part during dehydrogenation of eugenol and in part combined with the precursor during hydroxylation of the aromatic nucleus.

Under experimental conditions catalytic decomposition of H_2O_2 is unlikely to have altered the peroxide level. Peroxidase substrates in general and certain phenols in particular depress or inhibit the action of catalase (Stern and Bird, 1952, Goldacre and Galston, 1952).

In several experiments, the quantities of eugenol and peroxide consumed during the reaction were determined. When changes in reactants were estimated roughly from peroxide supplied and lignin formed, a figure of 1—2 mmol. H_2O_2 /mmol. eugenol was obtained, but more accurate determinations were desired.

Two groups of pea root sections, each 1 gm. fresh weight were prepared, one placed in 25 ml. of buffered eugenol- H_2O_2 , the other into buffered eugenol alone. Two additional vessels contained no tissue, but eugenol and peroxide respectively. The entire 25 ml. of eugenol in buffer was diluted to 200 ml. with 75 percent ethanol, and the optical density at 282 m μ determined spectrophotometrically on an aliquot of the above solution diluted a further twenty-five fold. The peroxide-buffer mixture was also diluted to 200 ml. and H_2O_2 determined by iodide titration on 5 ml. aliquots acidified with H_2SO_4 . Vessels containing tissue were incubated for 15 hrs. at 24° C., tissues collected and lignin determined. Reaction mixtures were made up to volume for final eugenol and peroxide determinations.

The initial reaction mixture contained 0.503 mmol. of eugenol and 0.882 mmol. of peroxide. After 15 hrs., 39 mg. of lignin had been formed (40 percent of total dry matter); 0.333 mmol. of eugenol and 0.59 mmol. of peroxide had disappeared from the medium. The amount of eugenol consumed, 55 mg., exceeds the quantity of lignin formed by about 40 percent. It has already

Table 2. *Inhibitors of Lignin Formation.*

Substance	Concentration (M)	Extent of Inhibition	
		Lignin formation	Peroxidation of Pyrogallol
NaCN	10^{-3}	85—100 %	85—98 %
NaN ₃	10^{-3}	80	80—90
Pyrogallol	10^{-3}	90	—
Guaiacol	10^{-3}	30—35	5—10
Cysteine	5×10^{-3}	100	100
Ascorbic Acid	5×10^{-2}	75	Transitory
3-Indoleacetic Acid ...	10^{-3}	25—100	100
Hydrazine Sulfate ...	10^{-4}	Transitory	5—10
MnCl ₂	10^{-3}	Transitory	None

been noted however, that more than one product is formed in the oxidation of eugenol (Siegel, 1954). In the overall reaction 1.81 mmol. of peroxide were consumed per mmol. of eugenol utilized. No more than a general significance can be attached to this relationship until all reaction products can be accounted for.

Inhibition of lignin formation was obtained previously with cyanide and indoleacetic acid, but a variety of other substances can prevent or retard lignin formation (table 2). Many effective compounds inhibit peroxidase itself. These include azide, cysteine and indoleacetic acid (IAA). The variable degree of inhibition with IAA is associated with differences in tissue; the formation of lignin by *Elodea* was inhibited completely with 4×10^{-5} M IAA, but pea tissue was only partially inhibited by a concentration of 10^{-3} M.

Pyrogallol is a far more active substrate for peroxidase than eugenol. Pea root tissue supplied with H₂O₂ (pH 4.5, phenol and peroxide both 10^{-3} M) will oxidize 10—15 μ Moles of pyrogallol/mg. protein-N/30 sec. but only .033 μ Moles of eugenol. Further, the degree of inhibition depends on the concentration of eugenol, hence, the action of pyrogallol is probably competitive. The effect of guaiacol can be regarded similarly.

When hydrazine is added to a eugenol-peroxide reaction mixture, the characteristic yellow intermediate fails to appear for about 15 min. instead of developing immediately. If a reactive semiquinone is formed as has been suggested, inhibition by hydrazine may result from "trapping" of the intermediate. When the hydrazine has been completely reacted, the conversion of eugenol to lignin can proceed. The similar effect of Mn²⁺ probably results from reduction of the intermediate and oxidation of the manganous ion, a phenomenon like that described by Kenten and Mann (1950).

Lignin Precursors

Additional compounds, vanillin, eugenol monomethyl ether, coniferyl alcohol, coniferin, phenylacetic acid, tyrosine and pelargonidin chloride have now been tested as lignin precursors, bringing to 21, the number of substances surveyed. Lignin was formed readily from coniferyl alcohol (on the basis of color tests and solubility) but coniferin, its β -D-glucoside yielded lignin only after 15-20 hrs. preincubation with pea tissue before addition of H_2O_2 . Eugenol monomethyl ether, without free hydroxyl groups, was neither a peroxidase substrate nor a lignin precursor. Phenyl acetic acid also failed to be oxidized by peroxidase, although phenylacetaldehyde has been shown by Kenten (1953) to yield benzaldehyde and formate peroxidatively. Pelargonidin chloride, a representative of the flavonoids, was prepared from petals of *Pelargonium zonale*. It was oxidized readily by peroxidase but formed no lignin. Tyrosine, a hydroxyphenylpropane with an additional function in the side chain, was oxidized slowly to relatively insoluble melanoid pigments but also failed to produce lignin.

Either the absence of a phenolic hydroxyl (as in cinnamic acid) or the blockage of an existing one by methylation or glucosidization is sufficient to prevent lignin formation. The behavior of coniferyl alcohol and coniferin indicates the presence in the tissue of a β -glucosidase. Conceivably, the localization, specificity and activity of glucosidases in plant tissue may be of considerable importance in controlling the supply of free lignin precursor, hence of the lignification process itself (Freudenberg, 1952 and Higuchi *et al.*, 1953).

The role of peroxidase in polymerizing phenols other than phenyl propanes has a bearing on our understanding of lignin formation. Pyrogallol, a widely used substrate, is converted into purpurogallin, a tropolone (Maehly, 1949); guaiacol yields the tetramer, tetraguaiaquinone (Sumner and Somers, 1947). In the oxidation of catechol, peroxidases, like phenolases, form melanin-like products of high molecular weight. The products of many other enzymatic peroxidations are unknown or uncertainly characterized and our present knowledge does not enable us to predict accurately whether a given substrate will be degraded as in the case of tryptophane (Wiltshire, 1953) or phenylacetaldehyde, polymerized, hydroxylated as in the case of monophenol, or simply dehydrogenated like benzidine or quinol.

Vanillin, of importance here because of its relation to lignins, yielded at least four recognizable substances when reacted with peroxidase- H_2O_2 .

0.5 mmol. of vanillin and 1.0 mmol. of H_2O_2 were incubated with 250 mg. of pea root tissue in 25 ml. of buffer for 3 hrs. By the end of this period an intensely

yellow solution and gray-brown sediment had formed. In the absence of tissue or peroxide, only the water-soluble yellow product appeared. This yellow pigment was also soluble in diethyl ether, but the brown sediment was soluble in neither of these solvents. The sediment was fractionated into three groups soluble respectively in hot ethanol and hot chloroform (insoluble ethanol) and insoluble in both of these solvents. All fractions were soluble in *N*/10 NaOH, but insoluble in an equivalent amount of NaHCO_3 , showing them to be weakly acidic, presumably as a result of the presence of phenolic hydroxyl groups. Fraction 1 was prepared as a deep orange crystalline product, fraction 2 in the form of light brown crystals, and fraction 3 as an apparently amorphous brown powder. The composition of both ethanol- and chloroform-soluble fractions was within 0.1 percent of that for vanillin itself (calculated: C 62.3 percent, H 5.3 percent). Insoluble fraction 3 differed somewhat: C 60.3 percent, H. 4.8 percent. All groups were N-free. From 76.0 mg. of vanillin, 6.0 mg. of ethanol-soluble material, 3.6 mg. of chloroform-soluble material, and 6.9 mg. of residue were obtained. The water and ether-soluble fraction has not yet been examined.

Agreement in elementary composition and differences in solubility between vanillin and its peroxidation products point again to polymerization reactions. The diversity of products obtained from vanillin suggests that non-enzymatic processes may succeed the formation by peroxidase of a single, simple intermediate compound.

Extracts of conifers and angiosperms rich in essential oils containing hydroxyphenylpropanes will, of course, yield lignin-forming materials. It was of interest however, to determine whether or not non-aromatic non-woody species contained substances able to serve as lignin precursors in this system. Concentrated hot water extracts (200–500 mg. of material/ml.) were prepared and 1–2 ml. added to 0.5–1.0 mmol. of H_2O_2 in 25 ml. of buffer containing 1–2 mm. slices of embryonic hypocotyl from the Red Kidney bean. After 5–10 hrs., the slices were observed for deposition of pigments and tested for lignin.

One or more parts of 20 species were tested. Species containing lignin precursors included seeds or seed coats of *Phaseolus vulgaris* var. Red Kidney, *Medicago sativa* var. Grimm, *Soya max.* var. Biloxi, and *Vicia faba*. The fruit of *Gleditsia triacanthos* and petals of *Pelargonium zonale* also contained precursors. The pigment pelargonidin is not a lignin precursor, hence other substances in the petals were responsible for a positive result. Grains and leaves of a number of cereals failed to yield precursors as did the leaves of coleus and onion.

Seeds should be particularly satisfactory for surveys of lignin precursors among herbaceous species insofar as they provide relatively high concentrations of phenols and often lignin.

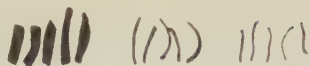


Figure 3. *Effect of synthetic lignin in pea sections on their shrinkage.* Left, eugenol- H_2O_2 , 51 %; center, eugenol only, 95 %; right, buffer only, 99 %.

The Effects of Lignin Deposition on Tissues

Throughout these investigations it has been observed that tissues containing eugenol-lignin differ from controls in physical properties. In addition to subjective characters, brittleness, ease of cutting, etc., the degree to which tissues shrink in volume on drying is a measure of their mechanical alteration. In one experiment, the volumes of 10 sections were measured from vessels containing respectively buffer only, eugenol, and eugenol plus H_2O_2 ; sections were then dried to constant weight at 100°C . and volumes redetermined. Shrinkage in volume is reduced considerably only for the tissue provided with both eugenol and peroxide (figure 3). This latter group lost 51 percent of its volume and the others 95 percent or more. Several experiments revealed a rough inverse proportionality between amount of lignin deposited and degree of shrinkage. In this instance, the lignin content of the group supplied the eugenol-peroxide mixture was 43 percent of total dry matter. Considering the localization of synthetic lignin in the cell wall, these findings can best be interpreted to mean that lignin has formed a «packing» material between the micellae and microfibrils of wall substance.

The correlation between natural lignin content and mechanical properties is apparent, of course, in the obvious differences in shrinkage during the drying of woody and non-woody plants or plant parts. As a further test of such correlation, a poinsettia stem (*Euphorbia pulcherrima*) was divided into internode segments and the weight and cross-sectional area of each segment determined. The summation of weight per unit area was computed as it varied from tip to base and converted into units of pressure-dynes/cm². The lignin content of each internode was determined by the sulfuric acid method. When the variation in pressure and lignin content is compared (figure 4), a close relationship is found along the stem except in the vicinity of the apex.

Reduced «wettability» is another physical property of tissues containing the synthetic lignin. This alteration is evident in the tendency of finely powdered tissue to float when in water or dilute acid for a considerable

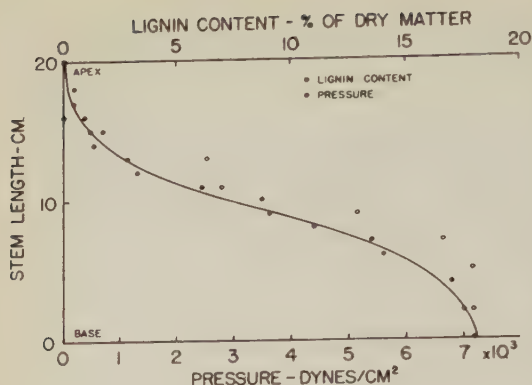


Figure 4. The relation between pressure gradient and lignin distribution in a *Poinsettia* stem.

period before sinking. Swelling of tissue slices in strong alkali is also noticeably reduced. Naturally lignified tissue is, of course, less permeable to water than non-lignified material. In nature the lignification process involves far more than increase in lignin content associated, as it is, with differentiation and a multitude of cellular changes. Nevertheless, lignin deposition has both ontogenetic and phylogenetic significance for the higher plant. Paech (1950) terms lignin formation the basis for the development of the higher plant and regards it as one of the most important skeletal materials in the plant. Bailey (1953) in treating of the evolution of vascular systems has related lignin in tracheary tissue to mechanical support of the plant body and has further associated little or no cambial activity with low forms, and high, sustained cambial activity with arboreal forms. It is perhaps of significance that such water forms as *Elodea*, possessing virtually no endogenous lignin, nevertheless retain the capacity for lignin synthesis, suggesting that the enzyme or enzymes involved in lignification are more widespread than lignin itself. Peroxidases may be found in fungi, algae, and thallose land plants. Hence if the evolution of the upright light plant did indeed depend on its capacity to synthesize lignin, it is in the phylogeny and genetic control of hydroxyphenylpropane production that the limitations on lignification will be found.

Summary

Continuing previous investigations on peroxidative lignin synthesis, eugenol synthetic lignin has been shown to be chemically similar in many of its properties to spruce lignin and to lignins in general: (a) the carbon, hydrogen and methoxyl contents are in close agreement; (b) behavior toward a wide variety of solvents is identical; (c) ultraviolet absorption spectra of

synthetic lignin and that from spruce especially are nearly superimposable; (d) the same color reactions are given by both natural and synthetic materials; (e) both natural and synthetic products are located in the cell wall and (f) both yield vanillin on nitrobenzene oxidation. Lignin synthesis from eugenol- H_2O_2 can be accomplished with washed cell wall fragments.

A visually observable sequence during the reaction of eugenol with H_2O_2 in the presence of pea tissue is strongly suggestive of the formation of a semiquinone as an intermediate in the reaction.

The efficiency of eugenol conversion is high in terms of yield and a ratio of 1—2 mmol. H_2O_2 /mmol. eugenol (as lignin) is indicated. Determinations of eugenol and H_2O_2 consumed during the reaction show an overall 1.81 mmol H_2O_2 /mmol. eugenol, however peroxidation products of eugenol other than lignin are formed.

Several inhibitors of lignin synthesis have been found, and although many of these inhibit peroxidase activity as measured with pyrogallol, some affect only the peroxidation of eugenol.

Further examination of precursors indicates that blockage of the hydroxyl group of eugenol, as in its methyl ether, or of coniferyl alcohol, as in its glucoside, prevent lignin formation. The glucoside is a suitable precursor if the aglucone is released by preincubation with the tissue, presumably through the medium of endogenous β -glucosidase.

Peroxidase may operate as a degrading, dehydrogenating, hydroxylating or polymerizing enzyme, depending on the particular substrate provided. The peroxidation of vanillin to polymeric substances has been described and discussed. Although not a lignin precursor, vanillin is an important substance in lignin chemistry and the diversity of products obtained by enzymatic peroxidation indicates that non-enzymatic processes play an important part in enzymatic peroxidations as secondary reactions.

Extracts of seeds and other non-woody parts of 20 species of plants show lignin precursors to present in some cases.

Finally, the mechanical consequences of lignin deposition show (a) that tissues containing eugenol lignin shrink far less on drying at 100°C . than do controls; (b) that the wettability of such tissues is also markedly reduced. These properties are also to be found in naturally lignified tissue. The close correlation between mechanical (pressure) gradient and lignin distribution is demonstrated using the poinsettia stem as an example. The significance of lignin in the ontogeny and phylogeny of the land plant is discussed briefly in the light of already existing concepts.

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References

- Andreae, W. A. & Andreae, S. R.: Studies on indoleacetic acid metabolism. I. The effects of methyl umbelliferone, maleic hydrazide, and 2,4-D on indoleacetic-oxidase. — *Can. J. Bot.* 31: 426. 1953.
- Adler, E. & Lindgren, B. O.: Some aspects of lignin structure. — *Svensk Papperstidning* 55: 563. 1952.
- Bailey, I. W.: Evolution of the tacheary tissue of land plants. — *Am. J. Bot.* 40: 4. 1953.
- Brauns, F. E.: The chemistry of lignins. — Academic Press, New York. 1952.
- Freudenberg, K., Lautsch, W. & Engler, K.: Die Bildung von Vanillin aus Fichten lignin. — *Ber. d. Chem. Gesellsch.* 73: 167. 1940.
- Reznik, H., Boesenberg, H. & Ranenack, D.: The enzyme system participating in lignification. — *Chem. Ber.* 85: 641. 1952.
- & Bittner, F.: Experiments with coniferyl alcohol containing radioactive carbon. — *Chem. Ber.* 86: 155. 1953.
- & Heel, W.: Di- und trihydroxycinnamyl alcohol. — *Chem. Ber.* 86: 190. 1953.
- Goldacre, P. L. & Galston, A. W.: The specific inhibition of catalase by substituted phenols. — *Arch. Biochem. and Biophys.* 43: 169. 1953.
- Higuchi, T., Kawamura, I. & Ishikawa, H.: On the formation of lignin in the bamboo shoot. — *J. Japanese Forest. Soc.* 35: 258. 1953.
- Kenten, R. H.: The oxidation of phenylacetaldehyde by plant saps. — *Biochem. J.* 55: 350. 1953.
- & Mann, P. J. G.: The oxidation of the manganous ion by plant extracts. — *Bioch. J.* 46: 67. 1950.
- Maehly, A. C.: Von Stand der Peroxydaseforschung. — *Z. Vitamin. Ferm.* 3: 115. 1949.
- Michaelis, L.: Semiquinones, the intermediate steps of reversible organic oxidation-reduction. — *Chem. Rev.* 16: 243. 1935.
- Paeckh, K.: Biochemie und Physiologie der Sekundären Pflanzenstoffe. — Springer-Verlag, Berlin. 1950.
- Siegel, S. M.: On the biosynthesis of lignins. — *Physiol. Plant.* 6: 134. 1953.
- Studies on the biosynthesis of lignins. — *Physiol. Plant.* 7: 41. 1954.
- & Galston, A. W.: Peroxide genesis in plant tissues and its relation to indoleacetic acid destruction. — In Press. *Arch. Biochem. & Biophys.* 1954.
- Sumner, J. B. & Somers, G. F.: Chemistry and Methods of Enzymes. — Academic Press, New York, 1947.
- van Fleet, D. S.: The distribution of peroxidase in differentiating tissues of vascular plants. — *Biodynamica* 113: 125. 1947.
- Wiltshire, G. H.: The oxidation of tryptophane in pea seedling tissues and extracts. — *Biochem. J.* 55: 408. 1953.

The Influence of Red and Infrared Light on the Respiration of Photoblastic Seeds

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Introduction

During the course of our investigations on the germination of lettuce seeds and its photoblastism (2, 3, and 4), it became clear that in order to clarify the mechanism of light action on germination it was necessary to study the respiration of germinating lettuce seed. Such a study would have to serve the following purposes:

- (1) To find out if the wave length which stimulates or inhibits germination has the same effect on respiration.
- (2) To study the time course of respiration from hour to hour in order to correlate the different stages of germination with different phases of respiration.
- (3) To find a basis from which the problem of the metabolic action of germination inhibiting and stimulating agents in general could be attacked.
- (4) To see if the different germination behaviour of freshly harvested seeds and seeds stored for different lengths of time could be related to changes in their respiration.

Besides these main aims the present paper contributes something to the much discussed question whether there is a respiratory response to light, and to our scant knowledge of the initial stages of the respiration of germinating seeds about which only very few data appear in literature.

Experimental Material and Methods

The seeds used in all experiments mentioned in this paper were lettuce seeds of the variety Grand Rapids which were harvested in 1950 in California, except for experiment e, the seeds for which were sown and harvested in 1951 in Jerusalem.

List of experiments.

Source of material	Date of experiment	Sign of exp. lot	Treatment	Sign of treatment	% germ.
P.W.	summer 1951 to autumn 1952	a	Seeds kept in darkness from beginning of imbibition till end of respiration measurement	D	24
»	»	b	after one hour of imbibition seeds exposed for 5' to blue light (Wratten filter 47)	IR	22
»	»	c	after two hours of imbibition seeds exposed for 5' to red light (Corning glass filter 245)	R	85
»	»	d	seeds decoated before imbibition. Otherwise as in Exp. a	D	67
Jerusalem	»	e	as in exp. a	D	12
P.W.	winter 1953/54	f	as in exp. a	D	34
»	»	g	after 15' of imbibition seeds exposed for 5 min. to infrared light (Wratten filter 88 A)	IR	24
»	»	h	as in exp. c	R	76
»	»	i	as in exp. c, but immediately after the red irradiation the seeds were exposed to infrared light as in exp. g	RIR	38

Before inserting the various light filters the white light source was adjusted to give 250 F.C. The filter used in experiment b transmits infrared as well as neutral blue light. As it was found that the inhibiting effect of this filter is due to the infrared light, this kind of treatment was designated IR together with the treatment described in Experiment g. The seeds were illuminated at different times after the beginning of imbibition according to the peak of the sensitivity they show towards the respective wavelength. During storage the seeds were kept air dry in linen bags at room-temperature in the dark. The experiments — germination and respiration measurements — were carried out at 26° C.

As is seen from the list above experiments f, g, h and i were carried out with the same seeds as a, b, c, d but after a considerably longer storage period.

The experiments were carried out as follows: 200 mg. of seeds and 0.25 ml. of water were placed into Warburg respirometer flask (average volume of the flasks 11—13 ml.). Only 100 mg. of seeds were used when working with decoated seeds (set d). 0.25 ml. of water were used (except d, where 0.15 ml. water was applied), after preliminary experiments had shown this to be the optimal amount. The open flasks were placed in tin containers having a water saturated atmosphere, which were transferred to an incubator at 26 °C. When the seeds were illuminated with R or IR the containers were taken out of the incubator, opened, illuminated and put back into the incubator. The respiration measurements were made in a circular Warburg respirometer by using the direct method with 0.2 ml. of 20 per cent KOH in the central well with a filter paper wick.

In order to carry out respiration measurements during for example, twenty eight hours, 112 lots of seeds were germinated. Every hour, four flasks were taken out of the incubator and transferred to the respirometer. Two bottles were used for CO_2 determination and two for O_2 determination. After an adaptation time of fifteen minutes the manometers were closed and the respiration measurement, which lasted for one hour, was begun. After this the flasks were discarded, and four new flasks were used for determining respiration during the next hour. Before the flasks were put into the Warburg respirometer, they were wrapped in dark plastic sheets, so that during the measurement no light penetrated to the seeds. The transfer from thermostat to manometer and the wrapping of the flasks were carried out in very weak neutral light which did not affect germination. Each experiment was repeated at least three times.

The respiration measurements for (a)—(e) were carried out every hour, whereas the respiration of (f)—(i) was measured every second hour. In order to test whether there was a difference, if the same respiration hour in the different experimental lots was measured on the same or on different days, the measurements were carried out in two ways: (a) respiration of seeds belonging to the same experimental lot, but germinated for two, three, four hours, etc., was determined during the same run of the respirometer, and (b) portions of seeds belonging to different experimental lots, but after identical time of germination, were measured at the same time. No significant difference was found.

Q_{O_2} and Q_{CO_2} were calculated as the amount of absorption or evolution of gas in microliters per 100 mg. air-dry seeds per hour.

Experimental Results

(1) *Experimental lots (a)—(e)*

Figures 1—3 give the Q_{O_2} , Q_{CO_2} and RQ measured. The diagrams show that during the whole course of respiration Q_{O_2} and Q_{CO_2} of the seeds kept in complete darkness (a) and of those treated with IR (b) are nearly identical. A statistical analysis has shown that from the third to the twenty eight hour the uptake of O_2 of (b) exceeds that of (a) by only 1.6 microliter per hour. This difference is statistically not significant at the 5 per cent level. The corresponding difference of CO_2 output is 1.8 microliter per hour, and this

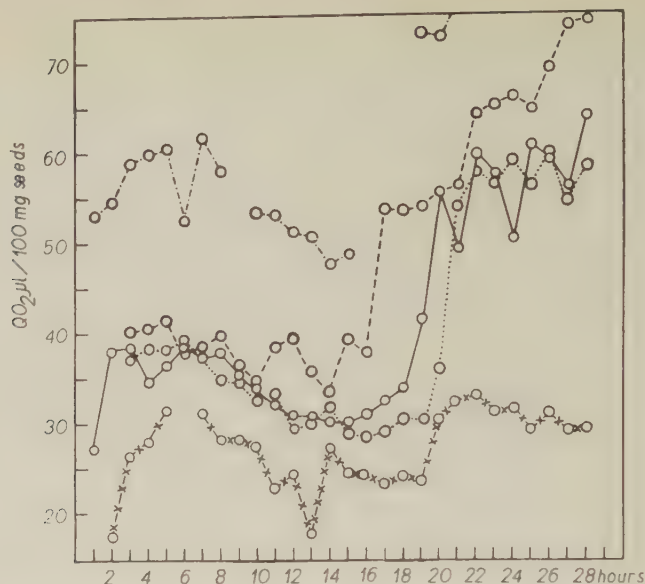


Figure 1. Effects of various treatments on the Q_{O_2} of lettuce seeds. Explanation see text.

—	exp. lot a	treatment D
.....	» b	» IR
-----	» c	» R
- · - · -	» d	» D
- x - x	» e	» D

difference is again not significant at the 5 per cent level. For the further analysis results obtained with (a) and (b) were therefore pooled, and are referred to as »darkness» (a—b). The curves of the seeds treated with R (c) are in general higher than those of the »dark» seeds (a—b). On an average, the uptake of O_2 of (c) exceeds that of (a—b) by 8 microliters per hour for the period from the third to the twenty eight hour. The corresponding figure for Q_{CO_2} is 6.6 microliter. Both these differences are highly significant ($P < 0.001$). These differences are partly due to the fact that the germination of seeds treated with R starts earlier than germination of »dark» seeds and those illuminated with IR. The steep second rise in oxygen uptake and carbon dioxide output is evident in all seeds, germinating and non-germinating ones alike, and coincides with the moment, where — in the germinating ones — the radicle emerges from the seed coat. This rise begins for the (c) seeds at about the fifteenth hour and for the (a—b) seeds at the eighteenth hour. The question, therefore, arose whether the difference in Q_{O_2} and Q_{CO_2} , observed between (a—b) and (c) for the whole period of twenty eight hours, is due only to the earlier start of germination of c, or whether the difference in

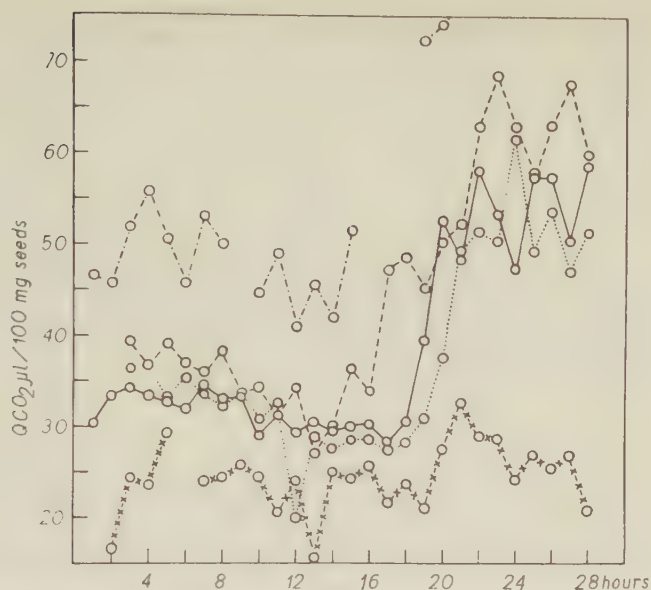


Figure 2. Effects of various treatments on the Q_{CO_2} of lettuce seeds. Explanation see text.

—	exp. lot a	treatment D
.....	» b	» IR
-----	» c	» R
-----	» d	» D
-x-x-	» e	» D

the rates of respiration is independent of the visible onset of germination. In order to answer this question the interval from the third to the twenty eighth hour was divided into two periods: the first extending until earliest germination observed in every lot, while the second starts, when the root emerges from the seed coat irrespective of the time at which this occurred. The levels of respiration are compared for the second period at corresponding time intervals after the respective start of root emergence.

For the pre-germination period the average rates of oxygen uptake and carbon dioxide output of lot (c) exceed the corresponding rates observed for lot (a-b) by 4.2 and 3.6 microliter per hour respectively. Both these differences are highly significant ($P < 0.001$). After the start of germination the differences are 3.5 and 2.9 microliter per hour. Both these differences are non-significant at the 5 per cent level. This means that the illumination with R has a significant effect on respiration rate only until the start of germination. During this period the stimulating effect of R on respiration is already evident one hour after illumination.

The freshly harvested seeds (e), which are much more dark dormant,

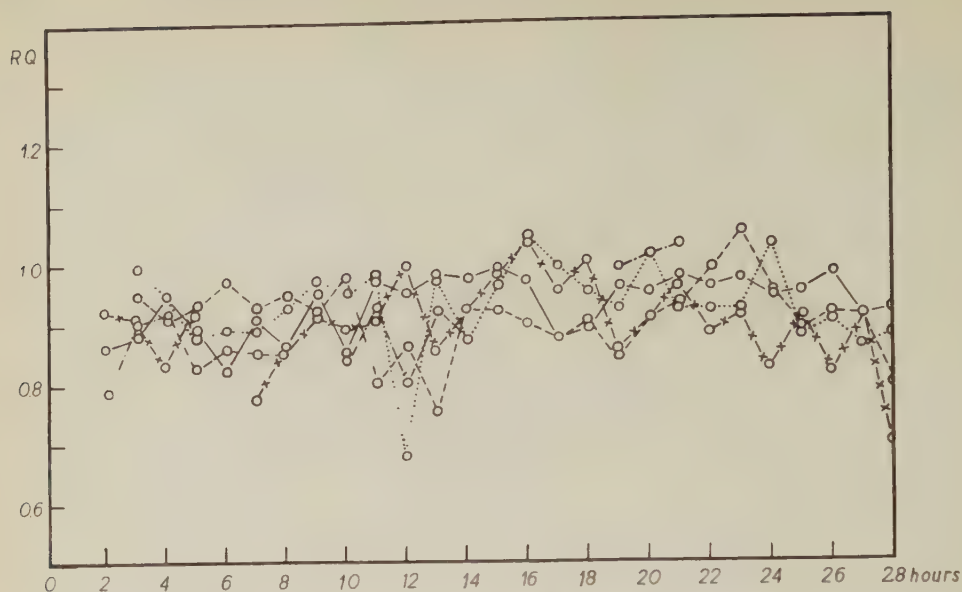


Figure 3. Effects of various treatments on the RQ of lettuce seeds. Explanation see text.

—	exp. lot a	treatment D
.....	» b	» IR
-----	» c	» R
- . - . -	» d	» D
- x - x	» e	» D

exhibit considerably lower rates of oxygen uptake and carbon dioxide output than the seeds (a—b) and (c). In contrast, the respiration level of the de-coated seeds (d) is already higher during the first hour after the onset of imbibition.

During the germination period in which measurements were carried out, respiration rates do not remain at the same level. With the start of imbibition the respiration rate rises to a first maximum which is reached during the fourth to sixth hour after the beginning of imbibition. The respiration rates of most lots then seem to decrease until the onset of germination (fifteenth to eighteenth hour), while respiration levels of the lots irradiated with R (curve c) suggest violent fluctuations of these rates. The minimum reached in all curves is followed by a sharp rise. The rise in the respiration rate is evident even for lots (e) whose germinations percentage is very low. In this case the rate of respiration after the short rise drops off slightly.

The statistical significance of the decrease of these rates was tested in two different ways:

Table 1. *Average respiration rates during the first and second parts of the pre-germination period.*

Gas exchange	lots	1 part	2 part	difference	P
Oxygen uptake	a	38.0	32.2	5.8	0.001
	b	38.2	31.2	7.0	0.001
	c	39.9	37.2	2.7	0.05
	d	57.9	51.9	6.0	0.01
	e	28.9	23.8	5.1	0.001
Carbon dioxide output	a	33.3	29.7	3.6	0.01
	b	33.8	27.5	6.3	0.001
	c	37.7	32.8	4.9	0.001
	d	50.4	45.6	4.8	0.05
	e	25.1	22.9	2.2	0.02

(a) By dividing the period before germination into two parts. All the respiration rates show a significantly higher level during the first than during the second part (the difference being significant at the 0.01 level; see Table 1), except Q_{CO_2} of (c) and Q_{CO_2} of (d) and (e), which are significant at a lower level only.

(b) By fitting a straight line to observed respiration rates, this trend decreased significantly with respect to all series. It might be mentioned that this slope was steeper with respect to seeds (a—b) than with respect to seeds (c), but this difference could not be shown to be statistically significant.

The RQ's of all seeds do not show any statistically significant differences (Figure 3). During the whole period of respiration measurements the RQ's remain more or less at the same level (about an average of 0.92).

(2) *Experimental lots (f)–(i).*

Figures 4–6 give the Q_{O_2} , Q_{CO_2} and RQ of lots (f)–(i). The most important fact is that throughout the period of measurement the respiration rate of the seeds treated with R (h) is very significantly higher than that of the seeds illuminated with JR (g). The Q_{O_2} and Q_{CO_2} curves of the seeds treated with RIR (i) occupy a more or less intermediate position between (h) and (g). The Q_{O_2} values of the dark seeds (f) do not differ significantly from those of the seeds treated with R (h) up to the eighteenth–twentieth hour, whereas the Q_{CO_2} values of the dark seeds are lower than those of lot (h). All the Q_{O_2} curves show a rise for the first hours, then remain at the same level up to the twelfth–fourteenth hour, and then rise considerably up to the twentieth hour. The h-curve goes on rising, whereas the other curves fall.

The Q_{CO_2} curves of (f), (g) and (i) fall to the eighth hour, then rise to the

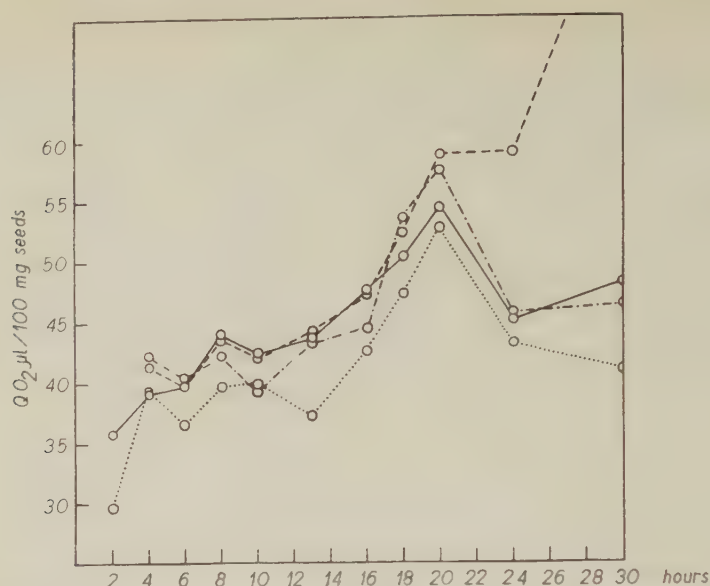


Figure 4. Effects of various treatments on the Q_{O_2} of lettuce seeds. Explanation see text.

—	exp. lot f	treatment D
.....	g	IR
----	h	R
-.-.-.-	i	RIR

sixteenth respectively twentieth hour, when a second fall sets in. The (h) curve does not show an initial fall and remains at the same level till the twelfth—fourteenth hour when it starts rising steadily.

The RQ values (Figure 6) fall considerably for the first eight hours, after which they rise slowly. During the whole period of measurement the RQ's of the seeds treated with R (h) are significantly higher than those of the «dark seeds» (f). The same is true for the seeds treated with IR (g) up to the thirteenth hour. The RQ values of the (h)-seeds and those of the seeds treated with R IR (i) are not significantly different.

A comparison between (a)—(c) and (f)—(i) shows the following differences:

(1) The Q_{O_2} values of (f)—(i) are higher than those of (a)—(c). From the sixth to the eighteenth hour onward things are reversed if we do not take into consideration the seeds treated with R.

(2) The Q_{CO_2} curves of (a)—(c) and (f)—(i) run more or less parallel and are at the same level up to the eighteenth till the twentieth hour. Then the values for (f), (g) and (i) drop to a much lower level than those of (a) and (b).

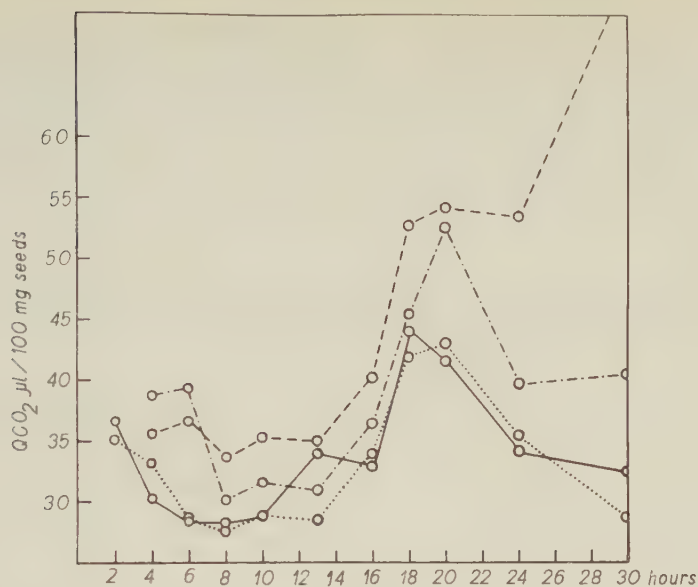


Figure 5. Effects of various treatments on the Q_{CO_2} of lettuce seeds. Explanation see text.

—	exp.lot f	treatment D
.....	» g	» IR
----	» h	» R
-.-.-.-	» i	» RIR

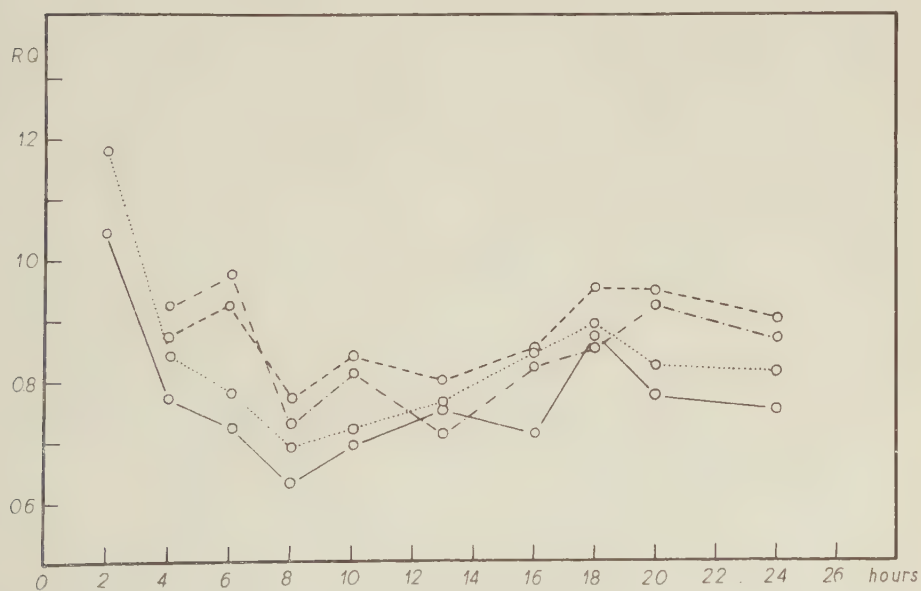


Figure 6. Effects of various treatments on the RQ of lettuce seeds. Explanation see text.

—	exp.lot f	treatment D
.....	» g	» IR
----	» h	» R
-.-.-.-	» i	» RIR

The RQ values for (a)—(c) do not differ from each other and remain more or less at the same level. In contrast to this there is a significant difference of the RQ values between R and dark, while the other RQ's are intermediate. The RQ's do not remain at the same level, but fall during the first eight hours and rise again.

Discussion

The most important fact brought out by this investigation is that germination promoting R and germination inhibiting IR have a pronounced effect on respiration.

With the experimental lots (a)—(c) R causes a rise in the respiration rates of the treated seeds, which means higher Q_{O_2} and Q_{CO_2} values throughout the experiment.

With the experimental lots (f)—(i) the stimulating influence of R on respiration is only evident on the Q_{CO_2} . This means that during storage a metabolic shift takes place inside the seeds causing a different respirational response to R.

The influence of IR on respiration changes with storage too. With lots (a)—(c) IR does not depress respiration below the values obtained from dark seeds. With lots (g)—(i) the Q_{O_2} values of IR-treated seeds are clearly lower than those of dark seeds. These changes in the effect of R and IR on respiration during storage are not astonishing as we know already that during storage the germination behaviour of seeds also changes. We take as example the fact that the germination percent of freshly harvested seeds is very low and rises with storage. The sensitivity of freshly harvested seeds to R is greater than that of old seeds. Freshly harvested seeds have a very low germination percentage in the dark.

A noteworthy and perhaps important point is the opposite influence of R and IR on the respiration of the old seeds. R raises the rate of Q_{CO_2} , IR depresses the rate of Q_{O_2} . R does not change the rate of Q_{O_2} and IR does not affect the rate of Q_{CO_2} . It is interesting that the influence of R and IR is not identical with that of IR alone, either in respiration or in germination. IR depresses the germination percentage below that of dark, R only to that of dark. This shows that influence of R cannot be cancelled completely by IR.

R retains part of its effect on respiration as the Q_{O_2} and Q_{CO_2} curves of R and IR are more or less intermediate between R and IR when given separately.

When we started our work there were only three papers by Schroepel (4), Kipp (9) and Leggatt (10) dealing with the influence of light upon the respiration of photoblastic seeds (*Nicotiana Tabacum* and *Lactuca*). Schroepel

and Kipp observed that the respiration curves of seeds kept in darkness show a rise coinciding with the maximum of imbibition followed by a decline. Light which stimulates germination stops the decrease of the respiration rate. Leggatt (10) found that blue germination inhibiting light depresses respiration by promoting a «carbon dioxide zymosis». After all the experimental work for this paper was done, a paper by Leopold and Guernsey (11) was published dealing with the influence of R and IR on respiration. They used the same variety of lettuce seeds (without stating their physiological age), but their technique of illumination was different. In their R treatment the seeds were continuously illuminated after one hour of darkness. In their IR treatment they gave the IR one hour only after R (one hour) using as control R-treated seeds. Q_{CO_2} values are not given, and their measurements cover only the first five hours of respiration. But even so, some common conclusions can be drawn. In both cases the effect of R and IR on respiration is already manifested a short time after the start of the irradiation and long before the first visible sign of the light effect on germination appears. This immediate response makes it probable that R and IR influence germination through their effect on respiration. In both cases R stimulated, IR depressed respiration.

But there is one difference: In our case the effect of R on Q_{O_2} is in the order of magnitude of 20—30 per cent, and there is no spectacular sudden rise. In Leopold and Guernsey's experiments the rise amounts to 660 per cent, is very sudden and subsides about one hour after the peak is reached. It may well be that these differences are due to the continuous application of R in their experiments on the one hand and our very short illumination times on the other. The fact that in their experiments when R was applied for only one hour the rise of the Q_{O_2} values was much smaller, tends to support this suggestion.

The respiration rate of decoated seeds which are nonphotoblastic and germinated in darkness with the same high percentage as in light (3), is from the beginning very much higher than that of complete seeds, even when these are treated with red light.

It is already known (3) that decoating has a pronounced influence on the respiration of various seeds. But the interesting point here is that by decoating the respiration rate rises to the same high level characteristic of the respiration of another lettuce seeds variety (Progress) which, under the temperature conditions used here, is not photoblastic, exactly like the decoated Grand Rapids seeds (Levari, 12).

The fact that freshly harvested, very photoblastic seeds have a much lower respiration rate than seeds stored for some time, suggest, in conjunction with the behaviour of decoated seeds, the following assumption:

Freshly harvested seeds possess a respirational block which can be eliminated by R and reconstituted by IR. In storage this block is partly overcome by slowly developing metabolic changes. This respirational block is the reason for the low germination percentage of the dark seeds. By decoating the seeds this block is removed. It remains uncertain if R removes the same or a different block, as the respiration values of the decoated seeds are higher than those of complete seeds treated with R. The respiration and therewith germination inhibiting block does not exist in non-photoblastic varieties such as Progress.

The physiological changes occurring during storage express themselves in three ways: There is a shift in the germination percentages in the dark, the response to R and IR is changed and the respiration is different.

This change in the respirational behaviour throws some light on the nature of the physiological shift involved. The average RQ's of a (0.928), b (0.928) and c (0.910) are higher than those of f (0.760), g (0.820), h (0.865) and i (0.846). These differences are in reality greater than a comparison of the averages can show. The values for (a)—(c) are for the whole time more or less at the same level without significant differences between the different curves. The values for (f)—(i) show distinct fluctuations during the period of measurement. We cite as example (g) which starts at 1.18, falls to 0.69 and rises again to 0.89. At the same time there are significant differences between the different curves of (f)—(i). The values for (f) for example are always significantly lower than the values for (h). This means that the different treatments applied to the old seeds used in (g)—(i) change the RQ's, which is not so for (a)—(c).

There are two possibilities which alone and together could explain the reason for the different average RQ values: During the storage the respirational system itself undergoes a change and runs after long storage along a different metabolic pathway. This possibility could be based on the fact that different treatments do not affect the RQ values of (a) — (c), but only change the respiration rate. But when applied to the old seeds, they change the RQ. Without being able to draw a final conclusion one could arrive at the following assumption: Young seeds possess a stable respiratory system. Sucrose is respired and the equilibrium between respiration and fermentation is fixed, does not change during germination, and is not affected by the different treatments. The respirational substratum of the old seeds is mixed and the equilibrium between respiration and fermentation is labile, changes during germination and is affected by the different treatments. It is interesting in this connection that Brown (1) observed rapid changes in the respirational activity and a rapid fall of the RQ in acorns during storage, accompanied by a rise in the germination percentage.

As far as the »young» seeds are concerned, there is good likelihood that sugar, i.e. sucrose, is the only respirational material used in respiration for the first twenty four hours. Poljakoff-Mayber (1953, 13) has shown that the »young» dry Grand Rapids seeds contain 400 mg. fat and 29 mg. sucrose per 1000 mg. of seeds. During the first twenty four hours of germination, i.e. the period of time in which we are interested here, the fat reserve remains wholly untouched, whereas the sucrose content decreases. The amount of oxygen absorbed by seeds germinated in light during this period is about 1200 microliter per 100 mg. of seeds. This is almost exactly the amount needed to oxidise the amount of sucrose lost during the corresponding time under the same conditions when calculated as mg. and corrected for NTP. RQ values are in good agreement with this.

It is interesting to compare our results obtained with a photoblastic variety of lettuce seeds with those worked out by Levari (12) working with the non-photoblastic variety »Progress». The rate of respiration of Progress is considerably higher. Only our decoated seeds approach the high values of Progress. The course of the respiration curves is very similar to that of (a) —(c). There is an initial rise, followed by a strong decrease, reaching its lowest point between the fourteenth and sixteenth hour. This decrease, just before the beginning of germination, is typical for all Levari's curves.

The average RQ of Progress is lower than the RQ's of (a)—(c) and (f)—(i), but the time-course of the RQ is very similar to that measured by us for (f)—(i). It falls from an initial 0.84 to 0.71 at the time of the general decrease of the respiration rate. This must mean that at the time of the decreasing respiration rate either there occurs a switch-over from one respirational substrate to another, or a change in the respirational pathway. Only additional research on the substrates respired and the part played by respiration and fermentation during germination can elucidate this interesting point. After the decrease, the RQ's rise again to 0.79 at the twenty fourth hour. This too happens more or less in a similar way with our lots (f)—(i). The RQ's of Progress are lowered by the germination inhibiting coumarin and 2,4-D which compared well with the fact that our dark (f) and IR (g) series, which both are characterised by inhibited germination, have a lower RQ than the R-treated seeds (h). Here, too, Levari comes to similar conclusions. She could prove that for Progress the O_2 absorption during the first twenty four hours is higher than the amount needed for the oxidation of sucrose present. Another substrate, besides sucrose, is respired. Coumarin and 2,4-D in her case, darkness and IR in ours, may disturb the equilibrium of these substrates, bringing about a change in the RQ. Another noteworthy feature of our respiration curves (a)—(e) and those of Levari is the initial rise followed by a second rise. This course of the curves cannot be compared with

the »great period of respiration» observed by Fernandez (8) and other authors. The »great period» is stretched over the first few days of germination, whereas in our case all the changes occur during the first twenty four hours. We have no explanation to offer. We can only state that the second rise cannot be explained by the rupture of the seed coat as it happens not only in those lots whose germination percentage is high but in the lots whose germination is very poor as well. The first rise coincides more or less with the maximum of imbibition. The depression before the second rise cannot be correlated with any known physiological events occurring inside the seeds.

Summary

The effects of germination stimulating red light and germination inhibiting infrared light were studied in photoblastic lettuce seeds var. Grand Rapids after different periods of storage. The following results were obtained:

(1) After a shorter period of storage:

Q_{O_2} and Q_{CO_2} of seeds illuminated with infrared light were almost identical with those of seeds kept in darkness.

Seeds treated with red light have a significantly higher Q_{O_2} and Q_{CO_2} till root emergence begins than seeds kept in darkness. This effect is already evident one hour after illumination.

The highest Q_{O_2} and Q_{CO_2} were found in decoated seeds kept in darkness.

Time curves of respiration rates show more or less the same pattern in all treatments.

RQ's remained at the same level and no significant difference could be found between those of seeds under different treatments.

(2) After a longer period of storage:

The effect of red and infrared light on respiration changes, as red light only raises Q_{CO_2} in comparison with darkness, whereas Q_{O_2} is not affected, while infrared light lowers Q_{O_2} only.

Seeds exposed first to red and afterwards to infrared light have rates intermediate between those of seeds treated only with red or infrared light.

The time curves of respiration rates differ from those after short periods of storage.

The RQ's do not remain at the same level and there are differences between those of different treatments.

It is suggested that in young seeds a respirational block exists which may be responsible for the germination behaviour of light sensitive seeds in darkness. This block may be overcome to various degrees by red light, by storage or by decoating the seeds.

We wish to express our sincerest thanks to the Pieters-Wheeler Seed Co., Gilroy, California, who as in previous investigations supplied the seeds free of charge. At the same time we thank Dr. H. Muehsam from the Hebrew University's Department of Statistics for his thorough statistical treatment of our material.

References

1. Brown, J. W.: Respiration of acorns as related to temperature and after-ripening. — *Plant Physiol.* 14: 621. 1949.
2. Evenari, M.: The germination of lettuce seed I. Light, temperature and coumarin as germination factors. — *Palest. J. Bot. Jerusalem* 5: 138. 1952.
3. — & Neumann, G.: The germination of lettuce seed II. The influence of fruit coat, seed coat and endosperm upon germination. — *Bull. Res. Council. Israel* 2: 75. 1952.
4. — — The germination of lettuce seed III. The effect of light on germination. — *Bull. Res. Council. Israel* 3: 136. 1953.
5. — — The germination of lettuce seed IV. The influence of relative humidity of the air on light effect and germination. — *Palest. J. Bot. Jerusalem* 6: 96. 1953.
6. — & Stein, G.: Factors modifying the influence of light upon germination. — *Nature* 172: 452. 1953.
7. — & Stein, G.: The influence of light upon germination. — *Experientia* 9: 94. 1953.
8. Fernandes, S.: Aerobe und anaerobe Atmung bei Keimlingen von *Pisum sativum*. — *Rec. trav. bot. neerl.* — 20: 107. 1923.
9. Kipp, M.: Die Abgabe von Kohlensäure und die Aufnahme von Sauerstoff bei der Keimung lichtgeförderter Samen von *Nicotiana tabacum*. — *Jahrb. wiss. Bot.* 71: 533. 1929.
10. Leggatt, C. W.: A contribution to the study of dormancy in seeds *Lactuca sativa* L. — *Canad. J. Res. C* 26: 194. 1948.
11. Leopold, A. C. & Guernsey, F. S.: Respiratory responses to red and infrared light. — *Physiol. Plant.* 7: 30. 1954.
12. Levari, R.: The effect of 2,4-D and coumarin on respiration of germinating wheat and lettuce seeds. — *Palest. J. Bot.* 6. 1953 (In press).
13. Poljakoff-Mayber, A.: Changes in metabolism of lettuce seeds during germination and its inhibition. — *Palest. J. Bot. Jerusalem* 5: 180. 1952.
14. Schroepel, F.: Katalase, Peroxydase und Atmung bei der Keimung lichtempfindlicher Samen von *Nicotiana tabacum*. — *Beih. Bot. Centralbl. Abt. 1*, 51: 377. 1933.

Factors Controlling Meristematic Activity in Excised Roots
VI. Effects of Various 'Antiauxins' on the Growth and
Survival of Excised Roots of *Lycopersicum*
***esculentum*, Mill.**

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Introduction

α -(1-naphthylmethylsulphide)-propionic acid (NMSP) at appropriate concentrations stimulates the growth of excised tomato roots cultured in a modified White's medium (Street, 1954). The concentration of NMSP required to produce maximum growth stimulation rises during repeated subculture of the main axis tip or with increase in the sucrose concentration of the medium from 2 to 3 per cent. The decline in growth and changes in root morphology which occur during repeated subculture of main axis tips (Street, McGonagle and Roberts, 1953) can be counteracted by appropriate NMSP treatment. These effects of NMSP have been explained on the hypothesis that it antagonises a naturally occurring root hormone which tends to *accumulate* to a supra optimal concentration during growth. Although this interpretation is supported by the demonstration by Aberg (1950) that NMSP is an anti-auxin, our results could not exclude the alternative hypothesis that NMSP stabilises or substitutes for some essential hormone which is *depleted* from the main axis meristem during culture. Ashby and Wangermann (1951) had earlier advanced a 'depletion' hypothesis of this kind to explain 'ageing' in *Lemna* and Wangermann and Lacey (1953) considered that this hypothesis was

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supported by their work with tri-iodobenzoic acid. This substance failed to counteract 'ageing' in *Lemna* although it antagonised the effects of added IAA. Alone it induced symptoms which they considered would result from a reduced effective auxin level. It was clearly desirable to see whether other substances having 'anti-auxin' activity would resemble NMSP in their effects on the growth of excised tomato roots and on the survival of their meristems when repeatedly subcultured.

A number of substances have now been tested for their effects on the growth of 1st passage tip cultures of excised tomato roots. These experiments were designed to examine whether the growth response to the substance under test takes the form of an optimum curve similar to that obtained with NMSP and whether this curve is shifted towards higher concentrations of test substance by increase in sucrose concentration of the medium. In appropriate cases this has been followed, according to whether the substance resembled NMSP or IAA in its growth effects, by assay of its ability either to enhance survival in 3 per cent sucrose medium (NMSP-effect) or to decrease survival in 2 per cent sucrose medium (IAA effect) (Street, 1954).

The substances tested are considered in two groups: —

Group I — Substances reported to have anti-auxin activity but differing markedly from NMSP in chemical structure: — 2,3,5-triiodobenzoic acid; α -(parachlorophenoxy)isobutyric acid, phenoxy-cyclopropane carboxylic acid.

Group II — Naphthyl compounds: — 1-Naphthoxyacetic acid; 2-Naphthoxyacetic acid, 1-Naphthaleneacetic acid.

Experimental

'Clonal' roots of *Lycopersicum esculentum* Mill (Best-of-All) were used throughout (Street and McGonagle 1953). Standard culture medium (Street and McGregor 1952) containing either 2 or 3 per cent sucrose and modified by addition of the substances under test has been used.

Group I substances

2,3,5-Tri-iodobenzoic acid (TIBA)

TIBA, tested in 2 per cent sucrose medium, failed to stimulate growth over the concentration range tested. At concentrations of 0.02 mg./l or below it was without significant effect. At 0.05 mg./l or higher concentration it was inhibitory to both main axis growth and lateral development (Table 1). In medium containing 3 per cent sucrose, inhibition was clearly evident at 0.02 mg./l and over the whole range the extent of the growth inhibition was

Table 1. *Effects of 2,3,5-triiodobenzoic acid (TIBA) on the growth of excised tomato roots.*
Each treatment represented by 18 replicate cultures.

Sucrose concentration g./100 ml.	Concentration of TIBA mg./l.	Increase in main axis length (mm.)	Lateral No.	Total length of laterals per root (mm.)
2	nil	56.1	23.6	107
	0.01	53.2	24.0	111
	0.02	54.3	20.1	115
	0.05	34.4	14.9	81
	0.10	25.8	6.5	43
	0.20	25.6	4.0	24
	0.40	22.9	2.1	12
	1.0	19.2	—	—
	2.5	no growth		
3	nil	42.6	13.4	78
	0.01	39.1	10.4	62
	0.02	24.5	8.2	57
	0.05	18.3	6.4	39
	0.10	15.2	2.9	20
	0.20	13.2	3.6	20
	0.40	12.6	2.1	14
	1.0	no growth		

increased by using the higher sucrose concentration. Survival of roots repeatedly subcultured in either 2 or 3 per cent sucrose medium was reduced by the presence of TIBA (Figure 1), although as with IAA the percentage growth inhibition (relative to the corresponding controls for each passage) did not progressively increase from passage to passage (Figure 2). Roots inhibited by TIBA resembled in morphology roots inhibited by IAA or by repeated subculture and the meristems ceasing activity in its presence were typical »sinks». TIBA had effects on excised root growth resembling those of IAA rather than of NMSP.

The form of the curve obtained by plotting TIBA concentration against increase in main axis length (data from Table 1) resembles that obtained by Åberg (1953) using seedling flax roots. Åberg suggests that at low concentration TIBA inhibits growth by acting synergistically with the natural auxin of the root and that the flattening out of the inhibition curve at somewhat higher concentrations is due to a balancing of this synergism by an antagonistic effect. The latter, however, never becomes dominant because with further increase in TIBA concentration toxic effects, not specifically related to the auxin system, supervene. In considering the nature of this synergistic or auxin-like effect of TIBA Åberg points to a report (Galston, Bonner and Baker 1950) that TIBA inhibits IAA-oxidase. Åberg considers that a similar synergistic effect is encountered in the effect of low concentrations of TIBA on shoot growth and Snyder (1949) has pointed to the marked toxicity to shoot cells of high TIBA concentrations. The inability of this substance to

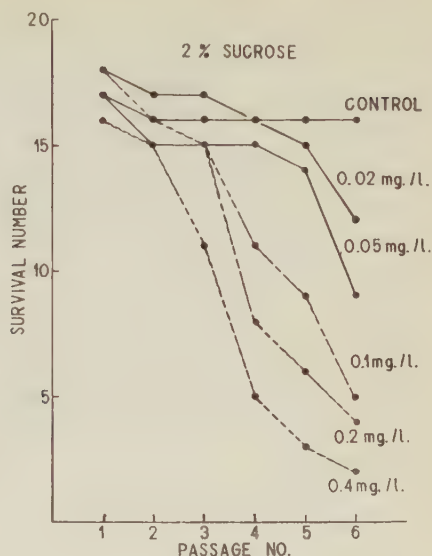


Figure 1. Effect of 2,3,5-triiodobenzoic acid (TIBA) on the survival of excised tomato roots in 2 per cent sucrose medium when repeatedly subcultured, at the end of each 7-day passage, by excision of the main axis tip. Concentrations of TIBA as mg. per l. of culture medium.

prolong the life of the *Lemna* frond (Wangermann and Lacey 1953) is therefore not surprising and its choice as an 'antiauxin' may have been unfortunate.

α-(parachlorophenoxy) isobutyric acid (PCIB)

This substance was found by Burström (1950) to increase the elongation of seedling wheat roots and this effect was explained as due to its antagonising the natural auxin controlling cell elongation. It also antagonised the inhibiting effect of externally applied IAA on the elongation of root epidermal cells.

The material used in the present experiment was synthesised by Professor H. Erdtman of the Royal Institute of Technology, Stockholm and was kindly made available to the author by Professor Burström.

The effects of PCIB on excised root growth in 2 and 3 per cent sucrose media is shown in Table 2. With 2 per cent sucrose there was increasing stimulation of main axis elongation with concentrations of PCIB up to 0.05 mg./l. although over the whole concentration range tested there was a progressive decrease in lateral number. Concentrations up to 0.05 mg./l. caused the lateral roots to be more widely spaced along the main axis but caused only slight retardation of lateral elongation (average length per lateral showed only a slight decrease). With 3 per cent sucrose the main axis elongation was not significantly affected by concentrations of PCIB up to 0.05 mg./l. though again lateral number was progressively depressed. Con-

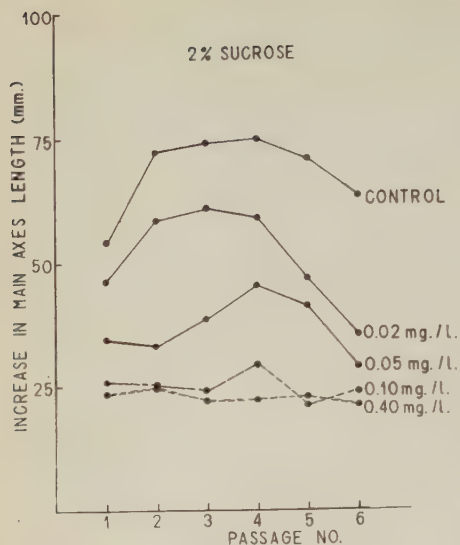


Figure 2. *Effect of TIBA on the mean values for increase in main axis length (mm.)* (Legend otherwise as Figure 1).

concentrations of PCIB above 0.05 mg./l. caused retardation of main axis elongation in both media.

Concentrations of PCIB up to 0.5 mg./l. did not significantly affect the survival of apices repeatedly subcultured in 3 per cent sucrose medium although the two highest concentrations employed (0.2 and 0.5 mg./l.) were inhibitory. Sensitivity to PCIB appeared to decrease with time and by the third passage there were no significant differences between the mean growth values for all PCIB treatments and the controls. A similar loss of sensitivity to PCIB was noted by Burström (1950).

Although PCIB stimulated main axis elongation in 2 per cent sucrose medium no similar effect was observed at higher sucrose concentration. The concentration at which inhibition of main axis elongation became apparent was unaffected by the sucrose concentration. Although there occurred a progressive decrease in lateral number over the whole of PCIB concentrations tested the rate of lateral elongation was not depressed until concentrations inhibitory to main axis elongation were reached. PCIB failed to enhance survival of roots repeatedly subcultured in 3 per cent sucrose. This substance therefore did not exert effects on excised root growth similar to those obtained with NMSP.

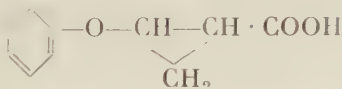
Burström (1950) concluded that the increases in root length caused by PCIB resulted from its stimulation of cell elongation. The increase in length of the root was entirely due to a greater length of the cells. PCIB was either without effect upon or depressed the rate of cell multiplication. Depression

Table 2. *Effects of α -(parachlorophenoxy)-isobutyric acid (PCIB) on the growth of excised tomato roots. Each treatment represented by 20 replicate cultures.*

Sucrose concentration g./100 ml.	Concentration of PCIB mg./l.	Increase in main axis length (mm.)	Lateral No.	Total length of laterals per root (mm.)
2	nil	59.4	29.1	153
	0.005	60.9	26.6	138
	0.02	66.4	23.1	109
	0.05	68.7	17.2	83
	0.10	57.1	9.1	38
	0.20	58.5	4.8	15
	1.0	31.2	—	—
3	nil	47.5	14.9	59
	0.005	47.6	13.2	61
	0.02	49.1	11.8	49
	0.05	48.3	7.4	35
	0.20	40.3	2.0	9
	0.50	34.7	0.5	2

of the rate of cell multiplication occurred at concentrations still markedly stimulatory to cell elongation. The effects of PCIB on cell elongation and cell division have not been examined on the present study but the wider spacing of laterals associated with PCIB treatment would be expected if the primary effect of this substance is on cell elongation. There is no evidence from Burström's work or our own that PCIB can stimulate meristematic activity. The hormone controlling cell elongation and which is antagonised by PCIB is presumably not that controlling meristematic activity.

Phenoxy-cyclopropane carboxylic acid (PCPC)



This substance was synthesised in the Station de Physiologie Végétale, Centre National de Recherches Agronomiques, Versailles and kindly made available to the author by Dr. G. Morel. It was found at that laboratory to have an effect on the growth of seedling barley roots similar to that recorded by Burström using PCIB on wheat roots (Private communication from Dr. G. Morel).

When tested against excised tomato roots growing in either 2 or 3 per cent sucrose media PCPC had the following effects: — (1) main axis elongation — no significant stimulation at concentrations down to 0.0002 mg./l. Inhibitory at 0.005 mg./l. or higher concentration. At concentrations from 0.05 to 0.50 mg./l. the inocula (10 mm) approximately doubled their length and only occasional roots produced laterals (1—3 in number). At concentrations above 0.50 mg./l. an increasing number of inocula were completely

Table 3. *Effects of 1-naphthoxyacetic (1-NOA) on the growth of excised tomato roots.*
Each treatment represented by 10 replicate cultures.

Sucrose concentration g./100 ml.	Concentration of 1-NOA mg./l	Increase in main axis length (mm.)	Lateral No.	Total length of laterals per root (mm.)
2	nil	63.8	28.1	121
	0.05	69.7	22.4	80
	0.10	64.0	21.0	88
	0.20	59.1	13.0	37
	0.50	29.0	—	—
	1.0	16.4	—	—
3	nil	45.8	13.8	63
	0.05	55.1	20.7	101
	0.10	58.2	18.9	91
	0.20	56.5	19.0	75
	0.50	39.6	3.5	8
	1.0	21.1	—	—

inhibited but remained white and floating. (2) lateral development — a progressive decrease in lateral number occurred with increase in PCPC concentration beginning at 0.005 mg./l. Mean lateral number was usually < 1 in presence of 0.05 mg./l. Lateral elongation was retarded at concentration causing retardation of main axis elongation (i.e. at concentrations above 0.005 mg./l.).

PCPC was either without effect or inhibitory to excised tomato root growth. The sucrose concentration of the medium did not significantly alter the response to PCPC. A similar depression of main axis elongation is caused by 0.005 mg./l. PCPC or 0.2 mg./l. PCIB. The toxic effect of PCPC is therefore manifested at a much lower concentration than that of PCIB and at no concentration did PCPC stimulate linear growth.

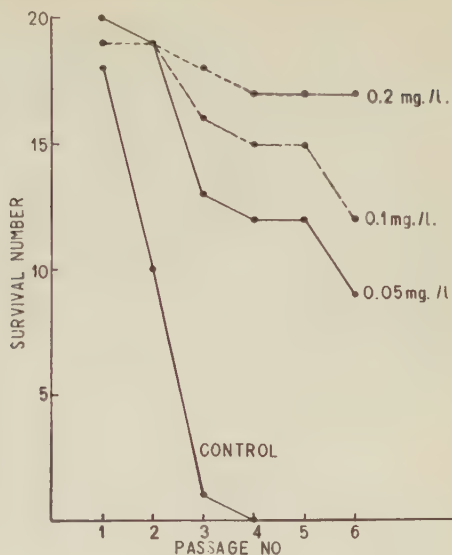
Group II substances

1-Naphthoxyacetic acid (1-NOA)

Dr. B. Åberg (private communication, 1953) suggested from his unpublished results that 1-NOA might have a similar 'antiauxin' action to NMSP.

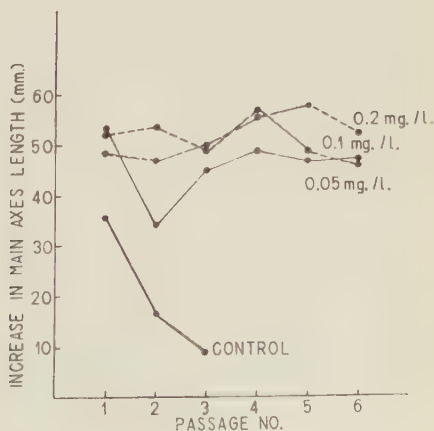
This substance has been tested for its effects on the growth of excised tomato roots over the concentration range 0.05 to 1.0 mg./l. In the concentration range 0.05 to 0.2 mg./l. it was either without effect or at some point produced a slight stimulation of main axis elongation when tested against roots cultured in 2 per cent sucrose medium. When tested against roots cultured in 3 per cent sucrose medium it produced marked stimulation of main axis elongation within this concentration range (Table 3). Even

Figure 3. *Effect of 1-naphthoxyacetic acid (1-NOA) on the survival of excised tomato roots in 3 per cent sucrose medium when repeatedly subcultured, at the end of each 7-day passage, by excision of the main axis tip. Concentrations of 1-NOA as mg. per l. of culture medium.*



at 0.05 mg./l. 1-NOA depressed the lateral number and total lateral length of roots in 2 per cent sucrose medium; this depression became progressively more marked with increase in the 1-NOA concentration. By contrast when roots were cultured in 3 per cent sucrose medium 1-NOA, at concentrations up to 0.2 mg./l., caused a marked improvement in lateral development. Further increase in 1-NOA concentration caused a similar suppression of lateral development to that noted in 2 per cent sucrose. In both culture media roots inhibited by high concentration of 1-NOA were thin, white and floating. The stimulation by 1-NOA of the growth of roots cultured in 3 per cent

Figure 4. *Effect of 1-NOA on the mean values for increase in main axis length (mm.) (Legend otherwise as Figure 3).*



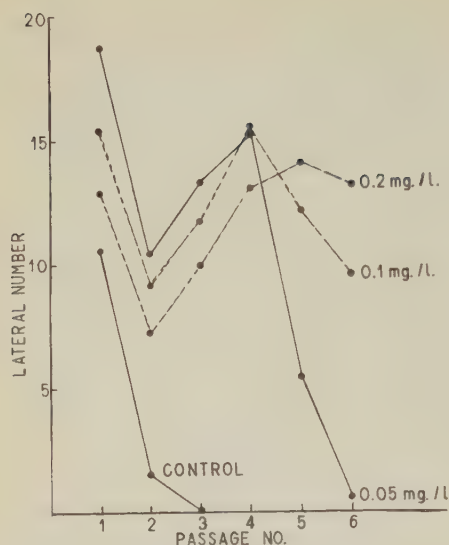


Figure 5. *Effect of 1-NOA on the mean values for number of laterals per root.* (Legend otherwise as Figure 3).

sucrose and the shift in the response curve to higher concentrations of 1-NOA resulting from increasing the sucrose content of the medium, clearly parallels the results previously obtained with NMSP (Street, 1954).

1-NOA also markedly enhanced growth and survival of roots repeatedly subcultured in 3 per cent sucrose medium (Figures 3, 4, and 5). Additions of 0.1 and 0.2 mg./l. 1-NOA to 3 per cent sucrose medium gave a higher general level of growth and better survival than had hitherto been obtained with NMSP. Though 0.05 and 0.1 mg./l. gave higher growth values in Passage 1 these concentrations were less effective than 0.2 mg./l. in maintaining growth (Figures 4 and 5) and survival (Figure 3). 1-NOA very effectively antagonised the decline and ultimate cessation of activity which otherwise occurs, particularly in 3 per cent sucrose medium, when excised root tip meristems are repeatedly subcultured. On the hypothesis previously advanced (Street, 1954) 1-NOA is to be regarded, like NMSP, as an antagonist of the root hormone which controls meristematic activity and which tends to accumulate to supra-optimal concentration during culture.

2-Naphthoxyacetic acid (2-NOA)

When tested against roots cultured in either 2 or 3 per cent sucrose medium, 2-NOA at concentrations of 0.001 or 0.01 mg./l. was without significant effect on root growth. Starting at 0.02 mg./l. it became progressively more inhibitory with increase in concentration (Table 4). There was no evidence of any stimulatory effect and roots treated with the higher con-

Table 4. *Effects of 2-naphthoxyacetic acid (2-NOA) on the growth of excised tomato roots.*
Each treatment represented by 10 replicate cultures.

Sucrose concentration g./100 ml.	Concentration of 2-NOA mg./l.	Increase in main axis length (mm.)	Lateral No.	Total length of laterals per root (mm.)	Notes
2	nil	67.7	40.7	137	
	0.001	64.7	39.9	130	
	0.01	64.4	40.0	148	
	0.02	49.8	29.2	107	
	0.05	30.9	5.7	25	
	0.10	20.8	3.5	11	Mean ¹ of 6 roots
	0.20	14.7	2.0	7	Mean of 4 roots
	0.40	24.0	—	—	Value for 1 root
3	nil	47.1	15.6	58	
	0.001	43.9	17.0	61	
	0.01	44.5	18.1	70	
	0.02	38.8	11.3	46	
	0.05	24.3	5.8	23	
	0.10	13.0	2.8	6.8	Mean of 6 roots
	0.20	19.0	4.0	16	Value for 1 root
	0.40	6.5	2.0	2.0	Mean of 2 roots

¹ Replicates not included in the mean showed no growth and were brown and sunken.

centrations were brown and sunken (contrast the effect of high concentrations of 1-NOA). Such roots resembled those inhibited by IAA. 2-NOA was quite different in its effects to 1-NOA, resembling in action IAA rather than NMSP.

1-Naphthaleneacetic acid (1-NAA)

1-NAA at concentrations of 10^{-10} g./ml. or lower is without effect on the growth of excised tomato roots cultured in standard medium. At higher concentrations it is inhibitory (Figure 6). In most tests 10–30 per cent of the roots are completely inhibited and sunken at 10^{-9} g./ml. and this is more marked at 2.5×10^{-9} g./ml. At 5×10^{-9} g./ml. almost all the roots are completely inhibited, brown and sunken. Increase in the sucrose concentration from 2 to 3 per cent does not markedly affect the response to 1-NAA except that at the higher sucrose concentration significant inhibition usually occurs at 10^{-10} g./ml. and the percentage inhibition is somewhat enhanced within the concentration range 2.5×10^{-10} to 2.5×10^{-9} g./ml. Results of a typical experiment are shown in Figure 6. In this figure increase in main axis length and lateral number for the 1-NAA treatments are expressed as percentages of the control values and any roots completely inhibited at concentrations 10^{-9} g./ml. or above have been omitted in calculating the mean growth values for these concentrations.

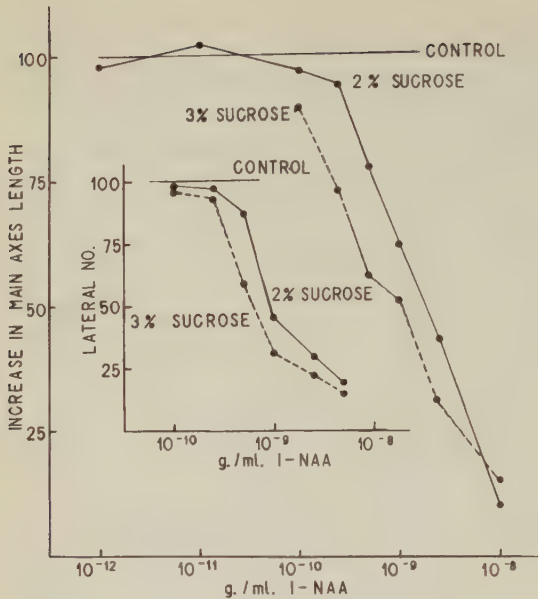


Figure 6. *Effect of 1-naphthaleneacetic acid (1-NAA) on the growth of roots cultured for 7 days in 2 and 3 per cent sucrose media.* Mean values for increase in main axis length and for number of laterals per root are expressed as percentages of the mean values for the control roots. Concentrations of 1-NAA as g. per ml. of culture medium.

Previous work (Street, 1954) had shown (i) that IAA has a deleterious effect on survival noticeable at concentrations which do not inhibit growth but more marked at inhibitory concentrations; (ii) that IAA at the threshold concentration for inhibition does *not* become inhibitory on repeated subculture and that at moderately inhibitory concentrations the inhibition does *not* become progressively more marked in successive passages. A similar experiment has now been undertaken on the effect of 1-NAA on the growth and survival of roots repeatedly subcultured in 2 per cent sucrose media (Figure 7). 1-NAA has, like IAA, a deleterious effect on survival even at concentrations which are not inhibitory to growth in the 1st tip passage. 1-NAA differs for IAA, however, in that concentrations (10^{-10} and 2×10^{-10} g./ml.) at the threshold of inhibition became inhibitory on repeated subculture. The onset of the depression of growth resulting from repeated subculture is accelerated in presence of these low 1-NAA concentrations. This progressively more marked growth depression is accompanied by an earlier onset of the fall in survival number and by the main axis of the roots thickening and becoming brown and brittle. These low concentrations of 1-NAA have therefore a cumulative effect which simulates, in a way which does not occur with IAA, an acceleration of the 'ageing' which occurs during repeated subculture in standard medium.

Evidence was presented (Street, 1954) to support the view that the effects

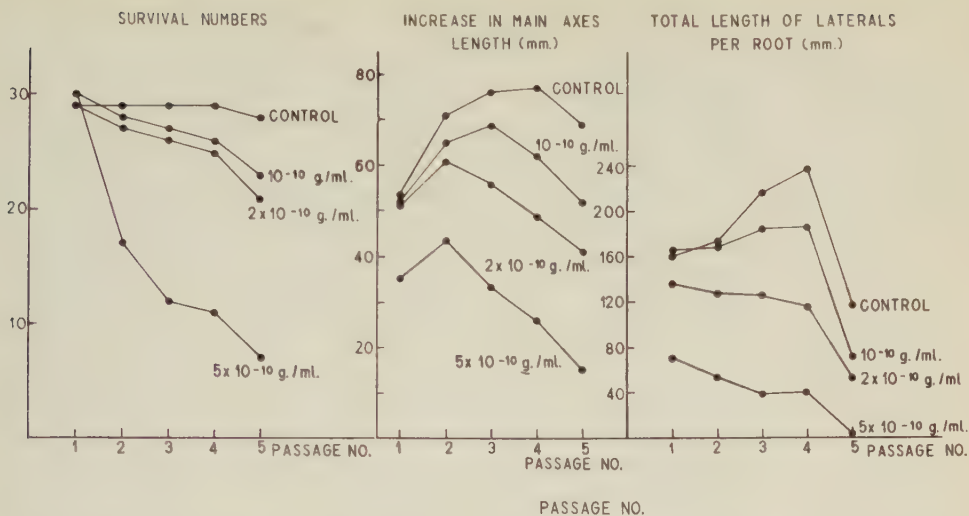


Figure 7. Effects of 1-NAA on the growth and survival of excised tomato roots in 2 per cent sucrose medium when repeatedly subcultured, at the end of each 7-day passage, by excision of the main axis tip. Concentrations of 1-NAA as g. per ml. of culture medium.

of NMSP on root growth and survival could not be explained in terms of an NMSP-IAA interaction. When 1-NOA was tested in a similar manner there was again no evidence, even at high IAA concentration (10^{-8} g./ml.) of any significant 1-NOA-IAA interaction. In view of the simulation of 'ageing' by 1-NAA and, in the hope of demonstrating the anti-auxin action of NMSP and 1-NOA, these substances have now been tested against roots inhibited by 1-NAA (Table 5). At appropriate concentrations both NMSP and 1-NOA cause a marked improvement in the growth of roots inhibited by 1-NAA. The results presented in Table 5 show that concentrations of 1-NOA which reduce growth alone are markedly stimulatory in presence of 1-NAA and that the most effective 1-NOA concentration is dependent upon the 1-NAA concentration. The antagonistic effect of both anti-auxins to 1-NAA inhibition is reflected in both main axis and lateral growth.

The facts that 1-NAA closely simulates the action of the hypothetical hormone postulated to control meristematic activity and that NMSP and 1-NOA, both of which enhance growth and survival, are also effective antagonists of 1-NAA, suggests that, when examining excised roots for natural auxins, tests should be devised which will detect on the chromatograms any naphthalene derivatives which may be present.

Table 5. *Effect of inhibition by 1-naphthaleneacetic acid (1-NAA) on the response of excised tomato roots to α -(1-naphthyl)methylsulphide)-propionic acid (NMSP) and to 1-naphthoxyacetic acid (1-NOA). Each treatment represented by 10 replicate cultures.*

Expt.	NMSP or 1-NOA additions	Increase in main axis length (mm.)	Lateral No.	Total length of laterals per root (mm.)	Increase in main axis length (mm.)	Lateral No.	Total length of laterals per root (mm.)	Increase in main axis length (mm.)	Lateral No.	Total length of laterals per root (mm.)
		Standard medium								
		Standard medium + 2.5×10^{-9} g./ml. 1-NAA								
(i)	Control	60.2	34.8	129	29.9	15.4	41			
	+ 0.25 mg./l. NMSP	64.2	15.0	34	49.9	26.7	62			
	+ 0.50 mg./l. NMSP	36.0	2.1	3	38.9	7.9	18			
	+ 0.75 mg./l. NMSP	23.1	—	—	27.8	4.3	6			
		Standard medium + 5×10^{-10} g./ml. 1-NAA								
		Standard medium + 10^{-9} g./ml. 1-NAA								
(ii)	Control	72.7	34.9	128	54.8	25.2	88	30.8	9.8	23
	+ 0.05 mg./l. 1-NOA	61.9	24.8	88	59.3	30.4	101	51.6	14.9	45
	+ 0.10 mg./l. 1-NOA	62.8	23.7	78	57.7	26.1	88	52.0	25.8	73
	+ 0.20 mg./l. 1-NOA	52.2	15.9	38	54.2	19.3	49	43.4	14.1	29

Summary

1. When tested over a wide range of concentrations 2,3,5-tri-iodobenzoic acid (TIBA), α -(parachlorophenoxy)-isobutyric acid (PCIB), phenoxy-cyclopropane carboxylic acid (PCPC) and 2-naphthoxyacetic acid (2-NOA) did not exert effects on excised tomato root growth similar to those obtained with α -(1-naphthylmethylsulphide)-propionic acid (NMSP).

TIBA and 2-NOA resembled in action β -indolylacetic acid (IAA) rather than NMSP.

PCIB stimulated main axis elongation in 2 per cent sucrose medium, but no similar effect was observed at higher sucrose concentration. The concentration at which main axis growth was inhibited by PCIB was unaffected by the sucrose concentration; even at inhibitory concentrations it did not significantly affect the survival of roots subcultured in 3 per cent sucrose medium. Sensitivity to PCIB appeared to decrease with time. No growth stimulation was obtained with PCPC and it was much more toxic than PCIB.

2. 1-naphthoxyacetic acid (1-NOA) stimulated the growth of roots cultured in 3 per cent sucrose medium. There was a shift in the response curve to higher concentrations of 1-NOA resulting from increasing the sucrose concentration of the medium. 1-NOA markedly enhanced survival of roots repeatedly subcultured. It had similar effects on growth, root morphology and survival to NMSP.

3. 1-naphthaleneacetic acid (1-NAA) inhibited excised root growth and, like IAA, had a deleterious effect upon survival. Low concentrations of 1-NAA applied to roots repeatedly subcultured in 2 per cent sucrose medium caused an acceleration of 'ageing'. Concentrations, initially without effect on growth, not only depressed survival but became progressively more inhibitory during repeated subculture. By contrast IAA does not show a similar cumulative effect at low concentrations.

NMSP and 1-NOA did not show any significant interaction with IAA but both exhibited a strong anti-auxin effect when tested against roots inhibited by 1-NAA.

References

- Åberg, B.: On auxin antagonists and synergists in root growth. — *Physiol. Plant.* 3: 447. 1950.
- On the interaction of 2,3,5-triiodobenzoic acid and maleic hydrazide with auxins. — *Physiol. Plant.* 6: 277. 1953.
- Ashby, E. & Wangermann, E.: Studies in the morphogenesis of leaves. VII. Part II Correlative effects of fronds in *Lemna minor*. — *New Phytol.* 50: 200. 1951.
- Burström, H.: Studies on growth and metabolism of roots. IV Positive and negative Auxin effects on cell elongation. — *Physiol. Plant.* 3: 277. 1950.

- Galston, A. W., Bonner, J. & Baker, R. S.: Flavoprotein and peroxidase as constituents of the indoleacetic oxidase of peas. (Abst). — *Amer. J. Bot.* 37: 677. 1950.
- Snyder, W. E.: Some responses of plants to 2,3,5-triiodobenzoic acid. — *Plant Physiol.* 24: 195. 1949.
- Street, H. E.: Factors controlling meristematic activity in excised roots. V. Effects of β -indolylacetic acid, β -indolylacetonitrile and α -(1-naphthylmethylsulphide)-propionic acid on the growth and survival of roots of *Lycopersicum esculentum*, Mill. — *Physiol. Plant.* 7: 212. 1954.
- & McGonagle, M. P.: Factors controlling meristematic activity in excised roots. IV. Habituation of the main axis meristem of excised tomato roots to repeated subculture. — *Physiol. Plant.* 6: 707. 1953.
- & Roberts, E. H.: Factors controlling meristematic activity in excised roots. II. Experiments involving repeated subculture of the main axis meristem of roots of *Lycopersicum esculentum*, Mill. and *Lycopersicum pimpinellifolium*, Dunal. — *Physiol. Plant.* 6: 1. 1953.
- & McGregor, S. M.: The carbohydrate nutrition of tomato roots. III. The effects of external sucrose concentration on the growth and anatomy of excised roots. — *Ann. Bot.* 16: 185. 1952.
- Wangermann, E. & Lacey, H. J.: Studies on the morphogenesis of leaves. IX. Experiments on *Lemna minor* with adenine, tri-iodobenzoic acid and ultra-violet radiation. — *New Phytol.* 52: 298. 1953.

Studies on the Browning and Blackening of Plant Tissues.¹
II. On the Interaction of Dopa and a Specific Oxidase
in the Leaves of *Stizolobium Hassjoo*

By

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1. Introduction

It was reported by Hattori and Kurihara (1) that the glycoside aucubin is the sole substance responsible for the blackening of the leaves, fruits and other parts of *Aucuba japonica* Thunberg, when they are injured, heated to about 60°, or fallen into necrobiosis. The process involved in the blackening of this plant is necessarily preceded by hydrolysis of aucubin by a β -glucosidase also present in the tissues, because aucubin itself is a very stable substance and possesses no free hydroxyl group. The aglycone, aucubigenin, which then becomes free, changes spontaneously and readily into a black substance by oxidation in the air. No oxidase was found in the leaves of this plant.

Now we wish to report another case of blackening of plant tissue brought about by the interaction of dopa (3,4-dihydroxyphenylalanine) and its oxidase. This plant is *Stizolobium Hassjoo* Piper et Tracy, a twining annual plant belonging to the family Fabaceae (Leguminosae). As is well known, dopa was first discovered by T. Torquati (2) in 1913 as an unidentified substance in the seeds of *Vicia Faba* L. and a little later M. Guggenheim (3) identified it with dopa which had been synthesized by C. Funk (4). In 1920,

¹ The title was changed so as to fit to the present and future direction of the studies.

² Née Michi Kurihara.

E. R. Miller (5) also isolated this amino acid from the seeds of a variety of velvet bean (*Stizolobium Deeringianum* Bort) and observed positive color reactions for dopa in other twenty six varieties of velvet bean, Yokohama bean (*Stizolobium Hassjoo*), and Lyon bean (*Stizolobium niveum* (Roxburgh) Kuntze).

We have been able to prove the presence of dopa, accompanied by several amino acids, in the leaves of *Stizolobium Hassjoo* by paper partition chromatography. When the leaves, stem, and root of this plant are injured, the color of the injured part immediately begins to change into brown and ultimately becomes black. Death-ring is also very easily formed on leaves. These color changes are accounted for by the oxidation of dopa by a polyphenoloxidase, which is also present in the leaves etc. It is also very interesting that the color change is more remarkable in younger leaves than in adult ones, and this is due to the fact that a greater amount of ascorbic acid is contained in the adult leaves than in the younger ones, because ascorbic acid prevents further oxidation of dopa beyond its quinone presumably by hydrating this back to the hydroxy compound.

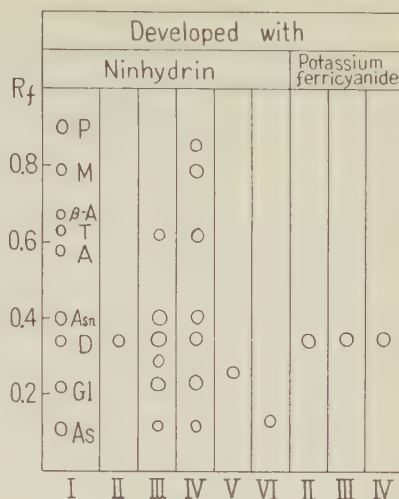
2. Proof of the presence of dopa

Fresh leaves of the plant collected in September last year were crushed in a blender with much cold ethanol, a procedure which prevents oxidation of dopa by an oxidase. Then the green mixture was filtered and the residue was several times extracted with cold ethanol to remove the last trace of dopa, the presence of which in the extract was easily detected by ferric chloride or potassium ferricyanide (pH 8.0 with phosphate buffer). The former reagent gives a green and the latter a red color. When no color was given with these reagents, the filtered leaf powder was dried in a vacuum desiccator, and the ethanolic extracts were condensed by distillation under diminished pressure on a water-bath. When the residue of the ethanolic extract, after dilution with water, was mixed with this leaf powder, it soon changed its color into dark brown. In this connection, it is to point out that the powder of younger leaves is more susceptible to darkening than that of adult leaves. This fact may depend upon the difference in the content of ascorbic acid, as shown later.

The ethanolic extract each of younger, pale green and adult, green leaves was paper-chromatographed. The solvent system chiefly used was 80 per cent phenol; butanol-acetic acid-water (4 : 1 : 1) was also used.

When developed with ethanolic ferric chloride, potassium ferricyanide (pH 8.0), or ninhydrin, respectively, a spot, green, red, or violet in color, respectively, was detected at R_f 0.34 (Figure 1) (80 per cent phenol as solvent),

Figure 1. *Chromatograms of leaf extracts.* I. Controls. P phenylalanine, M methionine, β -A β -alanine, T tyrosine, A alanine, Asn asparagin, D dopa, Gl glutamic acid, As aspartic acid. II. Watery exudate from the cut surface of youngest stem tip. III Younger, pale green leaves. IV Adult, green leaves. V Light brown, dying leaves on the lower portion of the stem. VI concentrated leaf extract after submitting to the action of leaf 'dopa oxidase'. The disappearance of all amino acids except one, which is to be regarded as aspartic acid, is very noteworthy. Details will be reported later.



which is much the same as that of authentic dopa. The presence of dopa in the leaves of various ages has been thus established. On examining the extract of light yellowish brown, dying leaves on the lowest portion of the stem, dopa was not found, but only a spot, which reacted with ninhydrin to give a violet color, was found at R_f 0.26 (80 per cent phenol as solvent). This spot has not, however, yet been characterized, but perhaps it may be glutamic acid, although the R_f -value is a little larger.

We have encountered an interesting fact, when we cut the very tip of the stem. There appeared a colorless watery drop on the cut surface. When smeared on a slide glass, this drop was dry after a few minutes, and white crystals separated, which showed characteristic forms under microscope. The crystals were dissolved in a small amount of water and paper-chromatographed as usual, when a spot corresponding to dopa was obtained at R_f 0.34. Other amino acids, which are usually found in plant extracts, could not at all observed (Figure 1). As is also to be seen from Figure 1, the pale green, younger leaves contain besides dopa and asparagin three amino acids, i.e. tyrosine, glutamic and aspartic acid, and the green, adult leaves methionine, tyrosine, glutamic and aspartic acid, and two unidentified amino acids except dopa.

3. Preparation and properties of oxidizing enzyme

The leaf powder, obtained by homogenizing 1 kg. fresh leaves in cold ethanol in a yield of 17.5 per cent, was extracted with a little more than

Table 1. Optimal pH for the oxidizing enzyme.

pH	3	4	5	6	6.8
Oxidation grade	30	52	80	96	100

1 l. cold water for 48 hours and squeezed through lint, followed by filtering with filter paper. There was obtained about 1 l. extract. Then 5 l. acetone were added to it and the white precipitate of the oxidizing enzyme was collected; yield about 3 g. This enzyme preparation was further purified by precipitating from its aqueous solution by adding acetone.

When a solution of 0.2 g. of the enzyme in 25 ml. water was mixed with an aqueous extract of the leaves of *Stizolobium*, a pronounced color change occurred after 10 minutes. After 24 hours, the black liquid was paper-chromatographed to see if any dopa is left intact. The result is, as shown in Figure 1, that all of dopa was entirely oxidized to form black substance. It is thus obvious that an oxidase capable of oxidizing dopa is present in the enzyme preparation.

The optimal pH for the oxidation of dopa by this oxidase was obtained by subjecting dopa ($M/1000$, in McIlvaine's buffer solution of pH 6.8) to the action of 1 per cent enzyme solution at 38° for 24 hours. The color produced was electrophotometrically estimated. As is shown in Table 1, the strongest oxidation took place at pH 6.8. At pH higher than 7, spontaneous oxidation of dopa readily took place.

Oxidizability of various phenolic compounds by this oxidase was tested by subjecting their $M/1000$ solutions (pH 6.8 with McIlvaine's buffer solution) to reaction for 24 hours at room temperature. The results are summarized in Table 2 as compared with the behaviors of tyrosinase and laccase hitherto known.

It is very interesting that *Stizolobium* oxidase oxidizes dopa, but does not attack tyrosine. In this respect, this oxidase resembles laccase, but does oxidize *p*-cresol. These findings seem to show that our *Stizolobium* oxidase is different from both of so-called tyrosinase and laccase and may represent an independent oxidase 'dopa oxidase'.

Inhibitory effects of various substances, known as inhibitors of oxidases, were tested in mixtures of 2 ml. McIlvaine's buffer solution, 1 ml. substrate solution ($10^{-2} M$), 1 ml. inhibitor ($10^{-3} \sim 10^{-2} M$) and 1 ml. enzyme solution (0.8 per cent) at 25° for 24 hours. As the substrates, tyrosine, dopa, and pyrogallol, and as the inhibitors, CO, 8-hydroxyquinoline, benzoin oxime, KCN, KCNS, thiourea, phenyl thiourea, *p*-aminobenzoic acid, sulfathiazole, sodium diethyldithiocarbamate, and *p*-nitrophenol were used. Complete in-

Table 2.

Compound	<i>Stizolobium</i> oxidase	Tyrosinase from potato tuber (our own results)	Tyrosinase	Laccase
Dopa	+++	+		
Gallic acid	+++	+		
Chlorogenic acid	+	+		
Hydroquinone	+(reddish)	+	—	+
<i>p</i> -Phenylenediamine	+	+	—	+
Catechol	+	+	+	+
Guaiacol	—		—	+
Resorcinol	—		—	
Pyrogallol	+	+	+	+
Rutin	—	—		
Tyrosine	—	+	+	—
<i>p</i> -Cresol	+(reddish)	+(reddish)	+	—

hibition was observed in every case except *p*-nitrophenol. Among these inhibitors, *p*-nitrophenol is noteworthy in that it inhibits oxidation of tyrosine by tyrosinase of potato, while the oxidation of dopa by potato tyrosinase was not affected by *p*-nitrophenol, but that of tyrosine considerably inhibited.

4. Influence of ascorbic acid upon the oxidation

As mentioned above, it was observed that there exists a considerable difference in the grade or velocity of oxidation between younger, pale green laves and adult green leaves. The question, what is responsible for this fact, seems to be of no little interest. As a first step of access to the problem, we examined the activities of the enzyme preparations of younger, pale green leaves and adult, green leaves. Five g. each of enzyme preparations were extracted with 60 ml. water at pH 6.8 for 48 hours at room temperature.

Two ml. each of these enzyme solutions were mixed with 1 ml. of aqueous dopa solution and 1 ml. McIlvaine's buffer solution, and the color brought about by oxidation was electrophotometrically estimated. There was, however, no difference observed between the two. Even by experimenting with other concentrations of dopa no difference was observed.

Since there is no possibility left for either dopa or its oxidase, or for both, to be the cause of the above difference, we then thought of possible participation of ascorbic acid in this matter. Dopa may be dehydrated at the initial stage of oxidation by its oxidase giving rise to an orthoquinone, and this in turn may be re-hydrated by the reduced form of ascorbic acid, or otherwise ascorbic acid may inhibit further oxidation of dopa quinone. Any way ascorbic acid may play thus an important role in the present problem.

Table 3. *Influence of ascorbic acid upon the oxidation of dopa by means of oxidase.*

Expt.	Dopa	Oxidase	Ascorbic acid	Colour strength	O ₂ -uptake (μl.)
A	1 mg.	1 ml.	0	73	20
B	1 »	1 »	0.264 mg.	59	60
C	1 »	1 »	0.528 »	32	92
D	1 »	0 »	0	—	0

37°; 140 min. Buffered with McIlvaine's solution of pH 6.8.

Five g. each of younger, pale green and adult, green leaves were minced with 20 ml. of 6 per cent trichloroacetic acid plus some fine quartz sand. The brei was filled up with water to 35 ml. and 1 ml. out of it was taken for the estimation of reduced form of ascorbic acid by the method of Folin, with the result that younger, pale green leaves contain only 27 mg. of it per 100 g. fresh weight, while adult, green leaves contain 60 mg. per 100 g. fresh weight.

So as to see whether or not oxygen uptake will take place, when ascorbic acid added to a mixture of dopa and oxidase, we made following manometric experiments (Table 3).

It is thus evident that the higher the ascorbic acid concentration is, the less is the oxidation due to oxidase. At the same time, however, the uptake of oxygen took place, and the quantity of oxygen absorbed is roughly directly proportional to the quantity of ascorbic acid added, a remarkable fact, which might show the probable reduction of dopa quinone which is formed by the oxidation of dopa. This oxidation and reduction might continue as long as ascorbic acid is available; hence the more ascorbic acid is added, the greater is the oxygen uptake and the less is the color strength. Since there is no serious difference between the concentration and oxidative activity of dopa oxidase in the leaves due to their age, it must be concluded that the quantity of the reduced form of ascorbic acid is the determinative factor for the oxidation of dopa in the leaves of *Stizolobium Hassjoo*.

5. Discussion

Dopa is hitherto known to be present only in plants of two genera of Fabaceae. Those are *Vicia* and *Stizolobium* (*Mucuna*). Of the former genus only one well-known species *Vicia Faba* contains dopa, but of the latter genus there have been recorded several species to contain it. As we have found, the cut surface of the youngest part of the stem excretes transparent watery sap which contains dopa as the sole amino acid or easily oxidizable polyphenolic compound. Dopa is readily oxidized in alkaline solution by atmospheric oxygen, but though somewhat stable in acidic medium, it under-

goes oxidation by an oxidase present in the same plant. This enzyme does not oxidize tyrosine, but on the contrary, tyrosinase of potato tubers oxidizes dopa. Hence this oxidase is to be considered as an example of so-called dopa oxidase; as ordinary tyrosinase prepared from potato has been said to oxidize dopa besides tyrosine, tyrosinase is either contaminated with dopa oxidase and difficultly separable from this, or tyrosinase is capable of oxidizing tyrosine as well as dopa. At present we do not aim at discussing the individuality of our 'dopa oxidase' or its relationship with tyrosinase, though the problem seems very attractive, but we shall return to this subject at another opportunity.

The fact, that the oxidation by dopa oxidase of dopa to colored substance or substances is inhibited or retarded by the addition of ascorbic acid and that ascorbic acid is found in a considerable amount in the leaves may suggest the possible participation of these three agents in the oxidation-reduction relation in *Stizolobium* plant.

That dopa is widely distributed in *Stizolobium* plant and seems to be accompanied by a considerably small amount of tyrosine, is also noteworthy. From what substance dopa is formed in this plant, is an interesting problem to be solved. Dopa is usually said to be produced by oxidation from tyrosine *in vivo* (6). According to the unpublished data of A. Komamine and one of us (S. H.), which have been obtained by experiments with etiolated seedlings of *Stizolobium Hassjoo* dopa seems to be derived from tyrosine.

6. Summary

1. It was proved that dopa is present in the leaves and young stem of *Stizolobium Hassjoo* as a substance most probably responsible for the blackening of the organs taking place on decaying or by injury.

2. Oxidation of dopa to black substance in *Stizolobium* plant is provoked by an oxidase, which does not oxidize tyrosine.

3. This oxidation of dopa to black substance, is inhibited or retarded by adding the reduced form of ascorbic acid, but exactly speaking, this inhibition may consist in either re-hydration of dopa quinone or in preventing further oxidation of this dopa quinone.

4. Younger, pale green leaves contain less ascorbic acid than adult, green ones, and the blackening reaction is more rapid and stronger on the former than on the latter. This may be ascribed to the difference in the contents of ascorbic acid (reduced form).

5. When the tip of the stem is cut, a drop of watery exudate appears on the cut surface. When dried, white crystals were formed and identified with dopa.

6. After oxidizing by oxidase the aqueous extract of the leaf powder, which was prepared by mincing fresh leaves with cold ethanol, dopa disappeared completely.

References

1. Hattori, Shizuo & Shiroya, Michi: Studies on death-ring. Part I. On an enzyme hydrolyzing aucubin in the leaves of *Aucuba japonica*. — Misc. Reports Res. Inst. Nat. Resources Nos. 17—18: 163. 1950.
2. Torquati, T.: Presence of a nitrogenous substance in the germs of the seeds of *Vicia faba*. — Arch. farm. sperm. 15: 213. 1913. (Cited after Chem. Abstr. 7: 2449. 1913.)
3. Guggenheim, M.: Dioxyphenylalanin, eine neue Aminosäure aus *Vicia faba*. — Z. physiol. Chem. 88: 276. 1913.
4. Funk, C.: Synthesis of *dl*-3:4-dihydroxyphenylalanine. — J. Chem. Soc. 99: 554. 1911.
5. Miller, E. R.: Dihydroxyphenylalanine, a constituent of the velvet bean. — J. Biol. Chem. 44: 481. 1920.
6. Raper, H. S.: The tyrosinase-tyrosine reaction. V. Production of *l*-3,4-dihydroxyphenylalanine from tyrosine. — Biochem. J. 20: 735. 1926.

Effects of Some Antiseptics on the Growth of *Chlorella*

By

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In the practical mass culture of *Chlorella* one of the troubles encountered is contamination by other microorganisms in the culture solution. With the object of finding an efficient antiseptic and also a growth accelerating substance for the cultivation of *Chlorella*, more than 300 experiments were made in The National Taiwan University, Taipei, Formosa, Free China. These experiments covered one whole year from August 1953. The following brief notes may summarize the results.

Equipment and Methods

A 20-liter glass bell jar was inverted and set on a wooden frame to be used as the culture apparatus. The light source consisted of 2 fluorescent tube-lamps of 40 watts and 2 incandescent lamps of 100 watts, which were placed 2 and 8 inches distant respectively from the lateral surface of the jar. A drain tube was fitted at the jar bottom. A tube for aeration was inserted from above and compressed air from a motor driven pump was blown into the culture solution. An automatically recording gasometer was connected to measure the volume of air passed through.

The period of aeration and lighting was nine hours a day. The volume of air passed (we used only air without additional CO_2) was 40 liters per hour for the 20 liters of culture solution. The seed cells were taken from a pure culture in test tubes. The basal culture solution consisted of KNO_3 1.5 gr. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 1.5 gr. KH_2PO_4 1.2 gr. and minor elements solution, to let the

basal culture solution contain in p.p.m.: Ca 30, B 20, Zn 20, Fe 10 and Mn, Mo, Cu 4, Co 1) tap water 1 liter. The initial pH was 5.5.

Other kinds of culturing apparatus were also used. There were porcelain and aluminum dishes of 12"×8"×2" in size. Each dish contained 2 liters of solution, lighted by one incandescent lamp of 100 watts which was placed 4 inches above the surface of the solution. The dish was also aerated at the same rate as the bell jar by passing air only. Two bell jars, 4 porcelain dishes and 10 aluminum dishes were operated continuously. After culturing for 5 days the cells were harvested by centrifuging, washing and drying. Also the average cell concentration per ml. was counted daily on the haemocytometer. During the culturing period, the pH of the solution was regulated to 6 by the addition of dilute sulfuric acid. Culturing temperature varied from 20° C to 28° C.

Species of Chlorella used

Chlorella pyrenoidosa, *C. ellipsoidea* and *C. vulgaris* were used separately. *C. vulgaris* (Pringsheim strain) always gave the best results.

Growth accelerating factor

The above-mentioned conditions are so poor for the growth of *Chlorella* that we consider it necessary to add some growth accelerating factors. While growth accelerators may not be needed under optimum conditions, their use as an emergency measure during poor weather or breakdown of the CO₂ supply, for instance, may be of appreciable supplementary importance in the operation of large-scale cultures. Although chelating agents have been reported to be useful for the acceleration of growth, various natural substances were tested in our experiments. At last we found that an extract of banana (*Musa sapientum*) gave the best results. The phenomenon that banana extract can promote the growth of *Chlorella* is surely not due to invert sugar alone. It may be that a certain growth factor is present in banana. By our long experience we know that banana extract is a good growth accelerating substance for *Torula* (we have studied for nine years the culture conditions of several species of *Torula* isolated from flower nectars). Thus with the addition of banana extract (10 grams of ripened banana meat in 100 ml. boiling water) 0.1 % of the basal culture solution, *Chlorella vulgaris* grows and vegetates very well under the normal aeration and lighting. After 5 days the net crop of cells (dry weight basis) per 20 liters amounted to 20 grams. The result is fairly good. As bananas are cheap and easily obtained in Formosa, the idea of the addition of banana extract to the basal culture solution may be of some economical importance to the mass culture of *Chlorella*.

Effects of antiseptics

In the practical mass culture of *Chlorella* the contamination by other microorganisms may cause a decrease of the crop. The contamination of animal origin is commonly attributed to *Paramecium*, *Vorticella* and mosquito pupae. Of plant origin, the contamination of *Bacillus subtilis* cannot be overlooked. Various antiseptics were added to the culture solution and tested for the prevention of contamination. The water extract of natural spices, such as white pepper, red pepper, ginger, onion, garlic and cloves were added separately to the culture solution in concentrations of 0.01 %—0.1 % of the material and found to be weakly active or entirely inefficient. Also, formaldehyde, phenol, β -naphthol, salicylic acid, sodiumsilicofluoride, acetic acid, ethyl alcohol, methyl alcohol, lemon-grass oil, Eucalyptus oil, and camphor were tested in different concentrations. Among these β -naphthol and salicylic acid were quite efficient. If the culture solution contained about 0.02 % β -naphthol or salicylic acid, *Chlorella vulgaris* grew well, while other organisms were greatly suppressed. The stock culture of *Chlorella vulgaris* in test tubes may be repeatedly transferred and cultured in banana extract-basal culture solution-agar containing an adequate quantity of β -naphthol or salicylic acid, so that the resistance can be gradually raised until the seed culture can tolerate 0.05 % of these antiseptics. It is interesting to note that only *Chlorella vulgaris* manifested the resistance while *Chlorella ellipsoidea* and *Chlorella pyrenoidosa* never did so. Whether the resistance against the antiseptics arises from a mutation or not is yet uncertain.

In conclusion the above enumerated notes that banana extract can be used as the growth accelerating factor and β -naphthol or salicylic acid can be used to prevent contamination may contribute some ideas to the future success of Mass culture of *Chlorella* in Formosa.

Mechanism of Action of Branching Enzyme from *Oscillatoria* and the Structure of Branched Dextrins

By

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The synthesis of branched polyglucosides in the blue-green alga, *Oscillatoria princeps* has been shown to be the result of a *branching enzyme* (8, 9, 10, 11). The function of this enzyme is the establishment of α -1:6 glucosidic linkages between residues. This is in contrast to *phosphorylase* which forms separate and distinct actions to this enzyme (17): the scission of an α -1:4 linkages. From preliminary studies (7, 10, 11), it would appear that the mode of action of the branching enzyme of *Oscillatoria* is similar to that of *isophosphorylase* (1, 2, 3).

Q-enzyme, the branching enzyme of potatoes, has been studied in some detail (15, 17). Peat et al are of the opinion that two possible mechanisms exist for the branching action of this enzyme. Both mechanisms relegate two separate and distinct actions to this enzyme (17); the scission of an α -1:4 linkage of a linear dextrin and the subsequent establishment of an α -1:6 linkage between the two split chain fragments. The original size of the linear dextrin for detectable Q-enzyme activity is claimed to be a minimum of 28 residues (17), and for rapid activity, 42 to 116 residues (15).

Both mechanisms make no mention of the need for glucose-1-phosphate as part of the substrates. It is the purpose of this paper to elucidate the action of the branching enzyme from *Oscillatoria*, and to offer a different mechanism for branching of linear dextrins by this enzyme.

Experimental

1. Preparation of Enzymes.

The preparation of branching enzyme was from the precipitate obtained with 0.8 saturated ammonium sulfate, as described (11). This precipitate was dissolved in 0.1 saturated ammonium sulfate and treated with solid ammonium sulfate until a concentration of 0.35 saturated was attained. The pH was adjusted to 6.5 with 0.1 *N* hydrochloric acid. The resulting precipitate was mainly branching enzyme. After 4 reprecipitations from 0.1 saturated ammonium sulfate, the material was free of phosphorylase activity. The precipitate was dissolved in sodium bicarbonate-manganese sulfate buffer (7) at a pH of 7.2. About 100 mg. of wet precipitate were dissolved in 20 ml. of buffer. Two ml. of this solution were used per 30 ml. of reaction mixture.

α -amylase was prepared from human saliva according to the variation of the method of Meyer et al. introduced by Whelan (22). Glucamylase was prepared by the method of Phillips and Caldwell (18). Amylo-1:6-glucosidase was prepared after Cori and Larner (5).

2. Preparation of Substrates

The maltodextrins were prepared from the acid hydrolysate of amylose after fractionation on charcoal-Celite according to the method of Whelan et al. (21). The maltodextrins isolated were maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose, and maltoheptaose (See Table 1). They were used in final concentrations of 0.1 Molar in the completed reaction mixture.

The dipotassium salt of glucose-1-phosphate (Schwarz Laboratories, N.Y.C.) was used in a final concentration of 0.05 Molar.

The reaction mixture for branching enzyme action was the same as previously described (7). The pH of the final incubation mixture with all components was adjusted to 7.2 (12). All incubation vessels were kept at 32° Centigrade.

α -amylase, glucamylase, and amylo-1:6-glucosidase were incubated with the material as described (22, 18, 5).

Table 1. R_f values of maltodextrins obtained by fractionation on charcoal-celite of products of amylose hydrolysis. (Phenol-water).

Maltodextrin	R_f value	
	Exptl.	Literature ¹
Glucose.....	0.40	0.39
Maltose	0.37	0.36
Maltotriose	0.31	0.305
Maltotetraose ...	0.265	0.25
Maltopentaose ...	0.22	0.20
Maltohexaose ...	0.19	0.18
Maltoheptaose ...	0.14	0.135

¹ After Whelan (21).

3. Methods of Operation

Identical 50 ml. flasks were set up containing reaction mixture, branching enzyme, a maltodextrin (from maltotriose through maltoheptaose), and dipotassium hexose monophosphate.

Samples were withdrawn every ten minutes and phosphorus was determined by the Fiske-Subarrow Method (6) with a Coleman senior model spectrophotometer.

The digests remaining after the action of branching enzyme had ceased, were heated to boiling for 2 minutes. The remaining glucose-1-phosphate was precipitated with Ba^{++} and the filtrate was divided into two approximately equal portions (about 10 ml.). One portion was incubated with α -salivary amylase, and the other portion with glucamylase. At the end of 24 hours, both digests were heated to boiling for 2 minutes, filtered, and 0.05 ml. portions withdrawn for paper chromatography. The remaining digests were then incubated with amylo-1:6-glucosidase for 6 hours at 35° C. At the end of this period, all digests were heated to boiling, filtered, and chromatographed.

Paper chromatography was carried out at 23° C, using Whatman No. 1 filter paper. Phenol-water was used for developing the chromatograms. At the end of 12 hours, the sugar zones were located by spraying with a mixture of 500 mg. benzidine, 10 ml. glacial acetic acid, 10 ml. 40 per cent trichloroacetic acid and 80 ml. of absolute ethyl alcohol.

Controls were set up containing reaction mixture, maltoheptaose, branching enzyme and no glucose-1-phosphate. Other controls were identical except that glucose-1-phosphate was included and the maltodextrin excluded. The pH and temperature of incubation were the same as for the experimentals.

Results

The maltodextrins, maltohexaose and maltoheptaose were branched by the branching enzyme of *Oscillatoria* in the presence of glucose-1-phosphate (Curves I and II, Figure 1).

There was no detectable activity of branching enzyme in the absence of phosphorylated glucose (Curve III, Figure 1).

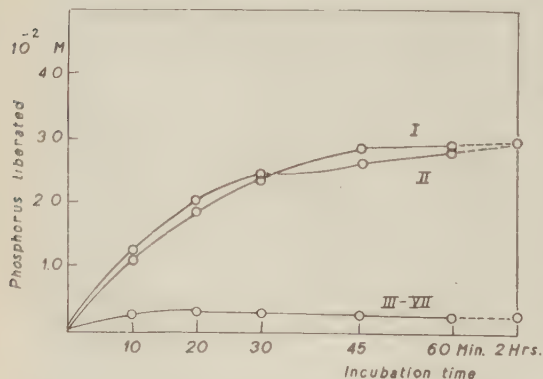


Figure 1. Reaction curves of branching enzyme. Curve I — presence of maltoheptaose; Curve II — presence of maltohexaose; Curve III — in absence of glucose-1-phosphate; Curves V, VI, and VII — with maltotetraose, maltotriose, and maltopentaose, respectively; Curve IV — control, without any type of maltodextrin.

There was no detectable activity with maltodextrins of shorter chain length than maltohexaose (See Curves V—VII, Figure 1).

The activity of branching enzyme in the presence of maltohexaose and maltoheptaose was about the same (cf. Curves I and II, Figure 1).

Branched dextrins were formed from these two maltodextrins as revealed by the enzyme degradation and paper chromatography studies in Tables 4 and 5.

Discussion

The branching enzyme of *Oscillatoria princeps* causes the synthesis of branched dextrins from the maltodextrins, maltohexaose and maltoheptaose, in the presence of glucose-1-phosphate. Actually, *two* substrates are involved in the reaction: glucose-1-phosphate, and a maltodextrin of the minimum chain length of maltohexaose. Previous mechanisms postulated for the action of the branching enzyme of potatoes, or Q-enzyme, make it necessary for that enzyme to act in two distinct and separate ways. These mechanisms are dependent upon the Q-enzyme splitting an α -1:4 glucosidic link in a linear dextrin, with the subsequent establishment of the split fragment in an α -1:6 position (17). This type of mechanism necessitates at least two active sites on the enzyme molecule. No need for glucose-1-phosphate is indicated as an associate substrate for Q-enzyme action (15, 17).



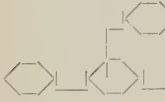
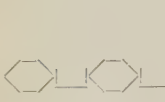
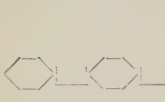
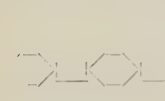
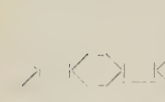
The mechanism offered in this paper would limit the branching enzyme of *Oscillatoria* to a single specific action, the establishment of α -1:6 linkages, and hence, necessitates but one active site on the enzyme molecule. The action of the branching enzyme then would be, the apposition of a glucose residue from the more highly energized phosphorylated ester in an α -1:6 position to one of the glucose residues in the maltodextrin.

It will be noted (cf. Figure 1), that maltodextrins of shorter chain lengths than maltohexaose are not utilized by the branching enzyme of *Oscillatoria princeps* for an associate substrate, and hence, no branching occurs. In other words, the smallest unit for detectable branching enzyme action appears to be a six residue α -1:4 configuration. This is in contrast to the reported sizes necessary for linear dextrins in the case of Q-enzyme (15, 17).

The actual point of branching, or position of the α -1:6 linkage, was elucidated by the enzyme degradation studies described (see Tables 2 and 3).

Table 4 shows the results of successive action of α -amylase followed by amyloglucosidase, and glucamylase (a type of maltase) followed by amyloglucosidase on the digest remaining after the action of branching enzyme of *Oscillatoria* on maltohexaose. The chromatograms revealed that maltohexaose and an unidentified product with a lower R_f value were present in


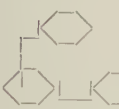

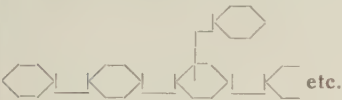


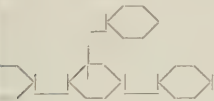
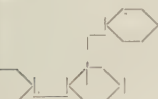
Table 2. Degradation studies of branched dextrin formed from maltohexaose by branching enzyme of *Oscillatoria*.

Possible positions of α 1:6 linkages	Final products detectable after:	
	α -amylase + glucosidase	glucamylase + glucosidase
 (no branching)	maltose maltotriose	glucose
 etc.	(1) glucose maltose	glucose maltohexaose
 etc.	(2) glucose maltotriose	glucose maltopentaose
 etc.	(3) glucose maltose maltotetraose	glucose maltotetraose
 etc.	(4) glucose maltose maltotriose	glucose maltotriose
 etc.	(5) glucose maltotriose	glucose maltose
 etc.	(6) glucose maltose	glucose

this digest prior to amylase action (see Columns 1 and 2 of Table 4). It will be noted that not all the maltohexaose had reacted. Some of the maltodextrin had reacted to yield a heavier molecule (as revealed by the lower R_f value).

After α -amylase had acted on this digest, three sugar spots were obtained, of which two proved to be maltose and maltotriose, and the third of which had an R_f of 0.24 (see Table 4). (Maltose and maltotriose can result as end-products of amylase action on maltohexaose (22, 23). Since there was some unreacted maltohexaose left in the mixture, this study did not in itself, reveal information as to the position of the branching. After treatment with amylo-glucosidase, a new spot appeared on the chromatogram, which was identified as glucose (see Tables 1 and 4). Since amylo-1:6-glucosidase is specific for terminal α -1:6 linkages (5), it can be assumed that there existed a glucose

Table 3. Degradation studies of branched dextrin formed from maltoheptaose by branching enzyme of *Oscillatoria*.

Possible positions of α 1:6 linkages	Final products detectable after:	
	α -amylase + glucosidase	glucamylase + glucosidase
 (no branching)	maltose maltotriose	glucose
 etc.	(1) glucose maltose maltotriose	glucose maltoheptaose
 etc.	(2) glucose maltose maltotriose	glucose maltohexaose
 etc.	(3) glucose maltotriose maltotetraose	glucose maltopentaose
	(4) glucose maltose maltotriose	glucose maltotetraose
	(5) glucose maltotriose maltotetraose	glucose maltotriose
	(6) glucose maltose maltotriose	glucose maltose
	(7) glucose maltose maltotriose	glucose

residue in this position somewhere in the molecule of the reacted maltohexaose (21).

It will be noted, that the sugar spots with R_f 's of 0.15 and 0.24 were no longer present in the chromatograms after glucosidase action. Hence, these spots must have been due to *branched* dextrans.

From this segment of the degradation studies, it can be assumed that branching occurred somewhere within the molecule other than off the third or fourth residues. If it occurred on either of these residues, *maltotetraose* would have been isolated from the digest after the action of α -amylase followed by amyloglucosidase (23) (see Table 2).

Table 4. Summary of results of enzyme degradation studies on the branched dextrin formed from maltohexaose. R_f values after chromatography in phenol-water.

Maltohexaose	After B.E. action	After α -amylase	After glucosidase	After gluc-amylase	After glucosidase
0.19	0.15	0.24	0.31	0.28	0.37
	0.19	0.31	0.37	0.40	0.40
		0.37	0.40		

The studies shown in Table 4 with glucamylase followed by amyloglucosidase, permit the exact localization of branching in the molecule. It will be seen that the final products as determined by paper chromatography (last two columns of Table 4), were glucose and maltose. If branching occurred on any residue other than the fifth in maltohexaose, *higher* malto-dextrins would have been isolated from the final digest (see Table 2).

The same analysis when applied to the digest from maltoheptaose, revealed *branching off the fifth residue* in this dextrin also (see Tables 3 and 5).

Hence, the branched dextrins formed by the branching enzyme of *Oscillatoria princeps* acting upon maltohexaose and maltoheptaose in the presence of glucose-1-phosphate, have an α -1:6 linkage branching off the fifth glucose residue. The product is interesting, in that after exhaustive phosphorylase action on a branched polyglucoside such as glycogen or amylopectin, a branched limit dextrin remains which has a similar configuration (5, 13).

The evidence would seem to indicate that the structure described is a basic one in all branched-chain polyglucosides. Also, it would appear that the action of amylo-1:6-glucosidase is the exact opposite of that of branching enzyme of *Oscillatoria princeps* (5).

Some indication of the structure of amylotic polyglucosides may be postulated from the evidence at hand.

The repetitiousness of a six unit structure in the polyglucosides seems to indicate that a six residue configuration is the basic structural unit of the

Table 5. Summary of results of enzyme degradation studies on the branched dextrin formed from maltoheptaose. R_f values after chromatography in phenol-water.

Maltohepta-ose	After B.E. action	After α -amylase	After glucosidase	After gluc-amylase	After glucosidase
0.14	0.12	0.22	0.26	0.27	0.31
	0.14	0.31	0.31	0.40	0.40
		0.37	0.37		
			0.40		

polyglucosides. For example, in amylopectin, a unit chain contains about 24 glucose residues (19). In glycogen, the unit chain has been reported to contain 12 or 18 residues (14, 20). The values appear to be indicative of a six-membered unit.

Further evidence for a six-membered basic unit may be obtained from paper chromatography studies (21). If the series of repeating polymers: maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose, and maltoheptaose, is chromatographed in phenol-water, the R_f values when plotted against the number of glucose residues per polymer, fall into a linear relationship from maltose through maltopentaose. *Maltohexaose* does not fall within this curve, but the relationship continues and is resumed from maltohexaose to maltoheptaose (21). (It would be of interest to study the relationship between maltoundecaose and maltoduadecaose). Since the «rate of flow» value in a solvent gives an indication of the molecular configuration, it can be assumed that some difference occurs between maltopentaose and maltohexaose, probably of a spatial nature.

The α -1:6 linkage is established by branching enzyme off the fifth glucosidic residue in a six residue maltodextrin; the same is true in a seven residue maltodextrin. The evidence presented, indicates that branching occurs off the fifth residue in both maltohexaose and maltoheptaose after the action of branching enzyme in the presence of glucose-1-phosphate. The fifth residue in these two maltodextrins exhibits a reactivity different from that of the terminal residue in maltopentaose. The reactivity of this fifth residue must be influenced, at least in part, by the presence of a glucose residue immediately following it as in maltohexaose and maltoheptaose. There is probably a morphological or structural difference between a 5 residue and a 6 residue dextrin other than merely the addition of another glucose residue. For example, maltohexaose and maltoheptaose are branched by the branching enzyme of *Oscillatoria princeps* in exactly the same manner and at approximately the same rate (see Figure 1).

It is suggested that the difference is due to the formation of a helix by maltohexaose and higher dextrans, while all dextrans below maltohexaose exist as a straight chain. It is a well established fact that assymetric molecules, when joined together so that every one has the exact same geometrical relationship in space to its neighbor, form a simple helix (16). The helical concept, as far as polysaccharides are concerned, is not new (14). This would indicate that the sixth residue in such a structure must be at the transition point of one spiral to another.

It is not necessary to assume that the suggested helix exists merely as a geometric result of bonding. The abnormal reactivity of the fifth residue in maltohexaose may be due to an electronic shift resulting from a rather weak

bonding joining the number one and number six glucose residues, forming a definite six-membered ring structure. This is further suggested by the abnormality in the increments in R_f values when glucose residues are added causing the chain length of five to be increased by one residue as indicated (21). It would seem that the activity of the fifth residue cannot be explained by mere stereo considerations. If steric hindrance were the important factor, then, since the fifth residue of maltopentaose is less hindered than the same residue in maltohexaose, the reaction rate with maltopentaose should be the greater of the two. The experimental evidence obtained in this study indicates that the exact opposite is true.

It would appear that the action of the branching enzyme from *Oscillatoria princeps* is in some way connected with the above mentioned electronic shift. This, in turn, suggests the possibility that this enzymatic action may be explained by the conventional type of electronic mechanism common to organic chemistry. Further work on this possibility is indicated.

Conclusions

1. The branching enzyme of *Oscillatoria princeps* will utilize maltohexaose and maltoheptaose as associate substrates, but will not branch the lower maltodextrins.
2. Branching occurs as a result of the apposition of a glucose residue from glucose-1-phosphate to the fifth residue of the maltodextrin. A mole of phosphorus is liberated at the same time.
3. It appears that the branching enzyme of *Oscillatoria*, by its synthesis of α -1:6 linkages, has the exact opposite action of amylo-1:6-glucosidase.
4. A helical structure is suggested for maltodextrins greater than maltopentaose.

The authors wish to thank Drs. Arthur Gentile, Duke University, Department of Botany, and Richard Klein, New York Botanical Garden Laboratories for their interest.

References

1. Bernfeld, P. & Meutemedian, A.: Isophosphorylase. — *Nature* 162:297. 1948.
2. — — Isophosphorylase and the formation of branched chain polysaccharides. — *Nature* 162:218. 1948.
3. — — Amylotic enzymes. I. Isophosphorylase. — *Helv. Chim. Acta* 31:1724. 1948.
4. Brown, F., Dunstan, S., Halsall, T., Hirst, E. & Jones, J.: Application of new method of end group determination to structural problems in the polysaccharides. — *Nature* 156:785. 1945.

5. Cori, G. & Larner, J.: Action of amylo-1,6-glucosidase and phosphorylase on glycogen and amylopectin. — *J. Biol. Chem.* 188: 17. 1951.
6. Fiske, C. & Subarrow, Y.: Colorimetric determination of phosphorus. — *J. Biol. Chem.* 66: 375. 1925.
7. Fredrick, J.: Preliminary studies on the synthesis of polysaccharides in the algae. — *Physiol. Plant.* 4: 621. 1951.
8. — A polysaccharide variant of *Oscillatoria princeps*. — *Physiol. Plant.* 5: 37. 1952.
9. — Induction of polysaccharide variants in *Oscillatoria* by low temperatures. — *Physiol. Plant.* 6: 96. 1953.
10. — Branching characteristics of *Oscillatoria* polysaccharides. — *Physiol. Plant.* 6: 100. 1953.
11. — Kinetics of polysaccharide formation in extracts of *Oscillatoria princeps*. — *Physiol. Plant.* 7: 182. 1954.
12. — »Enzyme content of plant tissues and organs» in *Handbook of Biological Data*, p. 82. (to be published by National Research Council).
13. Illingworth, B., Larner, J. & Cori, G.: Structure of glycogens and amylopectins. I. Enzymatic determination of chain length. — *J. Biol. Chem.* 199: 631. 1952.
14. Larner, J., Illingworth, B., Cori, G. & Cori, C.: Structure of glycogens and amylopectins. II. Analysis by stepwise enzymatic degradation. — *J. Biol. Chem.* 199: 641. 1952.
15. Nussenbaum, S. & Hassid, W.: Mechanism of amylopectin formation by the action of Q-enzyme. — *J. Biol. Chem.* 196: 785. 1952.
16. Pauling, L. & Corey, R.: Compound helical configurations of polypeptide chains: structure of the alpha keratin type. — *Nature* 171: 59. 1953.
17. Peat, S., Whelan, W. & Bailey, J.: Enzymatic synthesis and degradation of starch. XVIII. Minimum chain length for Q-enzyme activity. — *J. Chem. Soc.* 292: 1422. 1953.
18. Phillips, L. & Caldwell, M.: A study of the purification and properties of a glucose-forming amylase from *Rhizopus delemar*, gluc amylase. — *J. Am. Chem. Soc.* 73: 3559. 1951.
19. Potter, A. & Hassid, W.: Starch. I. End group determination of amylose and amylopectin by periodate oxidation. — *J. Am. Chem. Soc.* 70: 3498. 1948.
20. Schlamowitz, M.: On the nature of rabbit liver glycogen. I. Branching characteristics. — *J. Biol. Chem.* 188: 145. 1951.
21. Whelan, W., Bailey, J. & Roberts, P.: Mechanism of carbohydrase action. I. Preparation and properties of maltodextrin substrates. — *J. Chem. Soc.* 260: 1293. 1953.
22. — & Roberts, P.: Mechanism of carbohydrase action. II. Amylolysis of linear substrates. — *J. Chem. Soc.* 261: 1298. 1953.
23. — Enzymic breakdown of starch. — *Biochem. Soc. Symposia* 11: 17. 1953.

Spectrophotometrical Determination of Peroxidase in Living Roots

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Roots of cereals, of which wheat is one of the chief objects of this laboratory, contain comparatively large quantities of peroxidase, per volume tightly packed roots about 50 per cent of the concentration in horse radish tissue. The individual concentration of peroxidase, as of cytochromes, varies with the growth conditions. As shown in a previous communication (8) peroxidase participates only to a minor degree, if at all, in the aerobic respiration of wheat roots. Its presence, however, creates certain problems as to the spectrophotometrical observation of the oxidation-reduction state of the cytochromes, especially if effects of cyanide are studied. Because the quantity of peroxidase in the roots exceeds that of the total cytochrome system the γ -band at 404 m μ essentially contributes to the high elevation of the band in violet in the crude spectrum (3, 4). Because peroxidase is not reduced under anaerobical conditions this influence is eliminated if difference spectra, viz. ϵ reduced— ϵ oxidized, are calculated. Hence the absolute and relative values of the cytochromes may be calculated from such difference spectra. Disturbing effects may, however, be expected in studying the response of the cytochromes to cyanide and fluoride, azide and carbon monoxide, because these substances, according to Keilin and Hartree (1) convey considerable changes in the absorption spectrum of peroxidase. An approximate calculation of the difference spectra, viz. ϵ peroxidase · inhibitor— ϵ peroxidase, rendered bands in regions which are of special interest for the study of the cytochromes. In order to acquire more accurate difference spectra from the

two important complexes with cyanide and fluoride spectrograms from pure preparations of peroxidase were recorded in the authors automatic spectrophotometer.

Methods

By the courtesy of Prof. D. Keilin and Dr. E. F. Hartree the author received a sample of pure horse radish peroxidase in 0.5 *M* solution. For the spectrophotometrical work a small quantity, about 0.045—0.150 ml., was diluted to 0.530 ml., which is the volume of the Hilger micro-cuvettes used for the determination. The cuvettes are 3 mm. thick. For the dilution a solution of 0.02 *M* phosphate buffer, pH 5.6, was used, and this solution was also filled in the blank cuvette representing the reference beam.

The spectrum was investigated in the author's automatically recording spectrophotometer in intervals of 2 m μ (4). The instrument plots values of the sample and the reference beam on a logarithmically graduated chart, from which the extinctions can be directly read. At a slit opening of 0.01 mm. (in- and out-going slits alike) the band width amounts to only 0.25 m μ at 500 and 0.4 m μ at 600 m μ , a circumstance enabling very accurate readings, even at lower intervals than 2 m μ .

The cyanide complex was developed either by adding a small crystal of KCN to the cuvette, or by diluting the sample with 0.001 *M* KCN+0.02 *M* phosphate buffer. The spectrum is in both cases very constant. The fluoride complex was formed by diluting the sample with 0.1 *M* NaF at pH 3.3, or the same used for the study of the influence of fluoride on the cytochrome system (4). Contrary to the cyanide complex the fluoride complex develops more slowly, which can be noticed on the γ -band (see below). Observations were also made on the development of the H₂O₂ complexes of peroxidase. These will be separately published.

Difference spectra of peroxidase · CN and peroxidase · F

The γ -band of pure peroxidase appears at 404 m μ (according to Keilin and Hartree at 403 m μ) and that of peroxidase · CN at 424 m μ , resulting in a peak of the difference band at 428 m μ (figure 1). The peak of the corresponding difference band of peroxidase · F starts at 412 and moves back to 408 m μ in 30 minutes. According to Keilin and Hartree the peak of the fluoride complex is at 404 m μ . This would probably give an even lower position of the peak of the difference band than 408 m μ . The discrepancy is probably due to the time effect mentioned above. As a matter of fact the peak of the difference

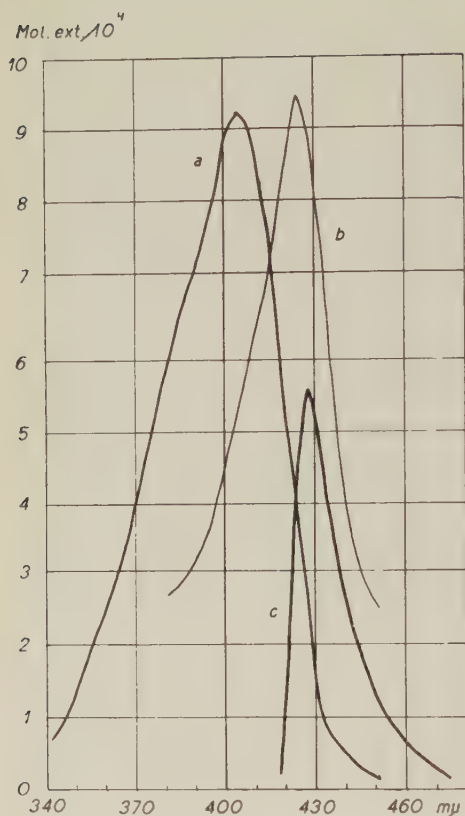


Figure 1.

Figure 1. Absorption spectrum of peroxidase. Curve *a*=pure peroxidase, *b*=peroxidase · CN, *c*=difference spectrum ϵ peroxidase · CN $-\epsilon$ peroxidase.

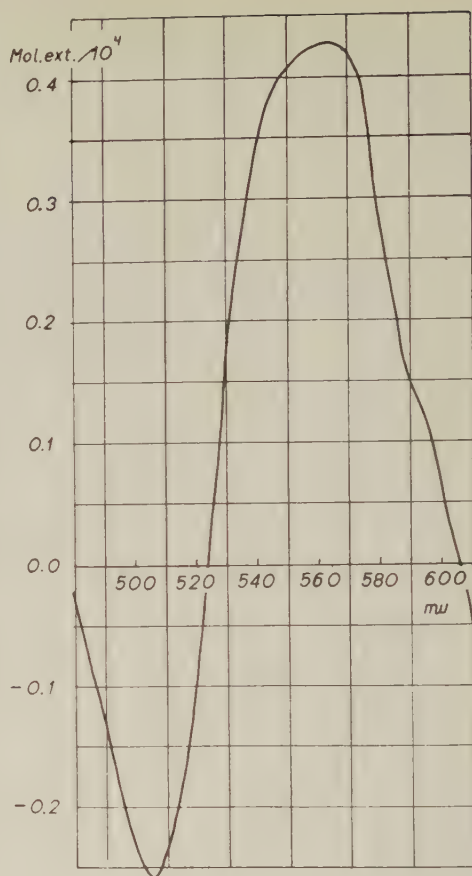


Figure 2.

Figure 2. Difference spectrum of ϵ peroxidase · CN $-\epsilon$ peroxidase.

spectrum of living roots treated with fluoride for 1—2 hours as a rule is somewhat lower than 408 mμ. These difficulties have little practical importance because the determinations of both the cyanide and the fluoride complexes in living material ought to be preferably performed on the bands in green or red respectively.

The prominent band of peroxidase · CN at 538 mμ, combined with the weak band of pure peroxidase at about 498 mμ, gives a large band in the difference spectrum, extended between the isosbestic points at 524 and 606 mμ and with a rounded peak at about 560 mμ (figure 2). This band

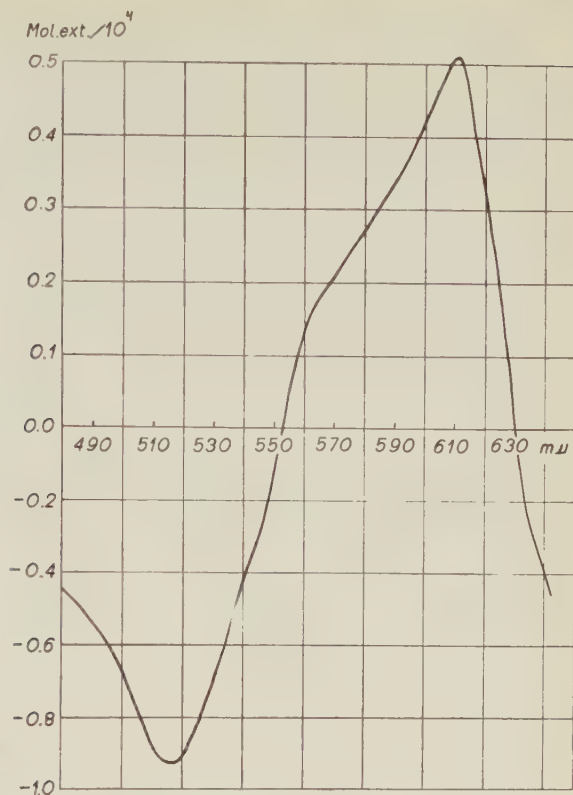


Figure 3. Difference spectrum ϵ peroxidase · F — ϵ peroxidase.

coincides with the reduced bands of the cytochromes *c*, *b*, and *dh* (cf. 6). A careful inspection of the top region of the band reveals a finer structure, but the differences in extinction are of an order not interfering with the measurements of the reduced bands of the cytochromes after inhibition with cyanide (see below). It is difficult to ascertain whether this fine structure is a property of the peroxidase · CN complex itself, or caused by minute quantities of desorganization products.

As shown by Keilin and Hartree (1) peroxidase · F shows a characteristic band at 612 mμ. The corresponding band of the difference spectrum (figure 3) has the same peak. It is steeply sloping toward red, with an isosbestic point at 630 mμ. The left side of the band is declining more gently, with a knick or elevation at about 562 mμ. The isosbestic point on this side appears at 554 mμ. A deep negative top appears at about 515 mμ. The considerable decrease of extinction of the difference band of peroxidase · F between 575 and 515 mμ has to be duly considered in computing the difference curves of living roots treated with fluoride.

Calculation of the peroxidase spectrum in living tissues

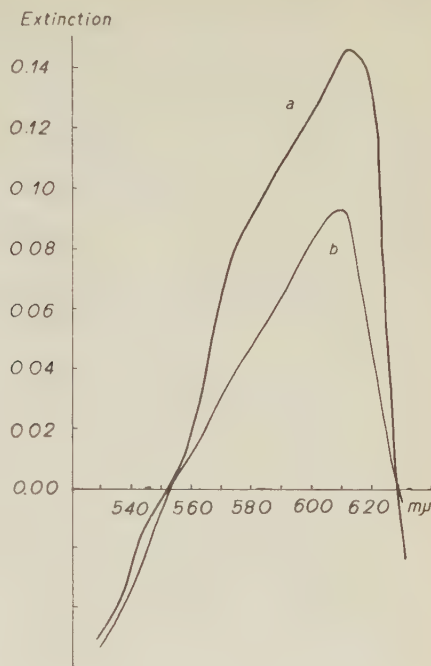
Because accurate determinations of the absorption spectrum of living material can only be made along the line of difference spectra (cf. 3, 4) an estimation of the concentration of peroxidase in the living cells has to be carried on via the fluoride or cyanide complexes. It is important to know, however, that the results can be corroborated by measurements of the pyridine haemochromogen spectrum, as will be shown below.

The appearance of the fluoride complex band at 612 m μ has been noticed by Keilin and Hartree and recommended by them for estimation of peroxidase in living tissues. The position of the band is indeed rather convenient, because the only cytochrome band in the neighbourhood is the reduced band of cytochrome oxidase at 604 m μ , which does not appear at fluoride inhibition (4), and which in addition to that is only weakly developed in roots. The difference spectrum of pure peroxidase \cdot F gives the value for molar extinction $\beta = 0.505 \cdot 10^4$, corresponding to the difference in extinction between 612 and 630 m μ . The band is clearly noticed in the difference spectrum of living roots of cereals and of horse radish (figure 4), if these are treated with 0.1 M NaF pH 3.3 for at least one hour.

Calculated from the molar extinction tightly packed roots of wheat contain on an average 9 μ mol peroxidase per litre. The corresponding value for slices of horse radish is 18 μ mol \cdot lit.⁻¹. Because wheat roots are cylindrical and do not fill the space more than up to 55–60 per cent the concentration of peroxidase per cell volume is, however, only little lower in roots as compared with horse radish.

The prosthetic group of peroxidase is the same as for the cytochromes *b* and *dh*, viz. protohaematin. This fact opens a possibility of corroborating these calculations with those made from the height of the α -band at 556 m μ of pyridine haemochromogen (molar extinction = $3.2 \cdot 10^4$). The roots were treated with a solution of 15 per cent pyridine plus some dithionite under anaerobical conditions for 24 hours and the bands of the pyridine compound accurately recorded in the automatic spectrophotometer. As shown elsewhere (6) the extinction at 550 m μ is only slightly increased by the presence of the pyridine compound of cytochrome *c* haematin, but the peak at 556 m μ is not measurably influenced. The extinction at 556 m μ is the sum of the values of peroxidase + cytochrome *b* + cytochrome *dh*. The apparent molar concentration of the cytochromes *b* and *dh* can be determined from the height of the reduced bands in the difference spectrum between roots surrounded by aerated salt solution and the same roots held anaerobically for 1–2 hours or longer. As shown in previous communications (4) the cytochromes are predominately oxidized (to about 80 per cent) in an aerated salt solution.

Figure 4. *Difference spectra ϵ peroxidase \cdot F— ϵ peroxidase from horse radish (a) and wheat roots (b). Thickness of the layers 14 mm. (for horse radish) and 16 mm. (for wheat roots).*



The values thus have to be corrected by c. 20 per cent to cover the total amount of protohaematin contained in cytochromes (the pyridine haemochromogen α -band of cytochrome oxidase appears at 585 mμ and does not interfere at all with the measurements).

As shown by the examples given in table 1 the values calculated from the peroxidase \cdot F compound and the net value of pyridine haemochromogen are in good agreement. In roots the pyridine values are as a rule somewhat higher, a fact probably caused by the very slow reduction of cytochrome *dh*, which gives not quite 100 per cent values for this substance.

The broad band of the peroxidase \cdot CN complex may be used for quantitative determinations, too, but only in combination with a careful analysis of the coincidence with the cytochromes, because at least cytochrome *c* is partly reduced in the presence of cyanide. The detailed investigation of the cyanide complex was undertaken mainly in order to find out the interference with bands of the cytochromes *c*, *b*, and *dh*. As will be shown in a later publication the cytochromes *b* and *dh* do not easily give complexes with cyanide at pH-values existing in the normal tissue. The extinction at 542 mμ in the difference spectrum, one of the isobestic points of cytochrome *c* (see 4), may therefore be used for the determination of the height of the peroxidase \cdot CN band in green. From this value the complete band can be cal-

Table 1. Comparison of determinations of peroxidase from the fluoride complex and the pyridine haemochromogen spectrum.

Tissue	Peroxidase. F $\mu\text{mol} \cdot \text{lit.}^{-1}$	Pyridine haemochromogen $\mu\text{mol} \cdot \text{lit.}^{-1}$		
		Total	Cytochromes <i>b</i> + <i>dh</i>	Peroxidase
Wheat roots	8.8	12.6	3.2	9.4
» »	5.7	9.0	3.2	5.8
» »	6.7	9.9	2.8	7.1
Horse radish	17.6	17.4	—	17.4

Notice. The values for roots are calculated per volume tightly packed roots (see above). No cytochrome has been detected in horse radish.

culated according to the values of molar extinction for each 5 $m\mu$ interval computed in table 2.

An average content of 8.8 μmol peroxidase per litre tightly packed roots develops a peroxidase $\cdot \text{CN}$ difference band of the height of $\epsilon=0.060$ at 560 $m\mu$, if the spectrum is recorded from a 16 mm. thick root bundle. For comparison it may be mentioned that the bands in the spectrum of completely reduced cytochromes from a similar bundle of roots amounts to the following values, computed from a series of 20 experiments:

Cytochrome <i>c</i> (550 $m\mu$)	Cytochrome <i>b</i> (563 $m\mu$)	Cytochrome <i>dh</i> (571 $m\mu$)
0.027—0.061	0.032—0.107	0.034—0.112
M: 0.042	M: 0.067	M: 0.072

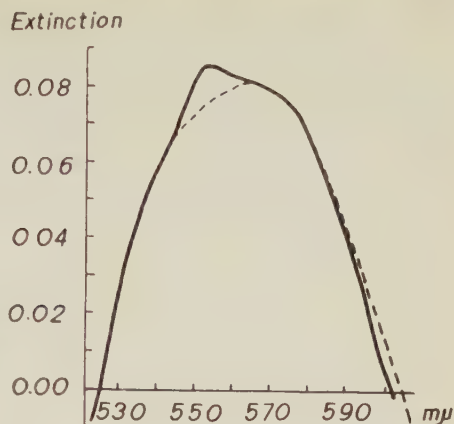
As mentioned above the values of cytochrome *dh* are probably somewhat too low.

The content of cytochrome varies considerably with the growth conditions (8). Fig. 5 shows one example of extremely low content of cytochrome, in which consequently the difference spectrum after treatment with cyanide is almost identical with the calculated curve of pure peroxidase $\cdot \text{CN}$. Only a slight elevation at 550 $m\mu$ here reveals the presence of some reduced cytochrome *c*. At higher concentrations of cytochromes the deviations from the peroxidase $\cdot \text{CN}$ spectrum are more prominent and also include elevations at 563 and 571 $m\mu$, corresponding to the partly reduced bands of the cytochromes *b* and *dh*.

Table 2. Molar extinction $\times 10^{-4}$ of the difference spectrum ϵ peroxidase $\cdot \text{CN}$ $-\epsilon$ peroxidase.

λ 520	525	530	535	540	545	550	555	560	565 $m\mu$
-0.090	0.007	0.112	0.269	0.333	0.386	0.409	0.427	0.430	0.428
λ 570	575	580	585	590	595	600	605 $m\mu$		
0.414	0.388	0.300	0.229	0.151	0.130	0.066	0.015		

Figure 5. *Difference spectra ϵ peroxidase · CN— ϵ peroxidase for wheat roots with low content of cytochromes. The dotted curve represents the calculated participation of peroxidase.*



The γ -spectrum of roots treated with cyanide

The γ -region is less suited for accurate measurements of bands of cytochromes because of the fact that these are present both in the oxidized and reduced state, only moved a distance varying between 10 and 24 $m\mu$ toward red at reduction. This fact certainly enables the computation of difference spectra, but the calculation is considerably hampered by the coincidence of eight single bands — one pair each of *a*, *c*, *b*, and *dh* — which are moving up and down in a narrow region of about 30 $m\mu$. To these eight bands are then added those of peroxidase and its cyanide and fluoride compounds. The band of pure peroxidase partly covers the ox- and red-bands of cytochrome *c* and *bd*, and the ox-band of *a* (at 420 $m\mu$). The displacement of the peroxidase band from 404 to 424 $m\mu$ after the addition of cyanide severely disturbs the observation of the above named bands of the cytochromes. Fluoride is far less disturbing.

The difference band of peroxidase · CN at 428 $m\mu$ (see figure 1) is narrowly situated between the reduced bands of *b* and *dh* at 424 and 431 $m\mu$. As these cytochromes are fairly resistant to cyanide the peroxidase · CN band at 428 $m\mu$ would be expected to appear in roots treated with cyanide. Observations of the CN-spectrum of roots, however, frequently show a large band with its peak at 436—440 $m\mu$ (fig. 6). This would at the first sight point to a modification of the wheat peroxidase, as compared with the horse radish preparations, a modification which would not affect the bands in green.

Observations of the development of the peroxidase · CN bands in solutions of increasing concentration, starting with 10^{-8} *M*, and observations of the time course in 10^{-4} *M* solutions, show a very rapidly proceeding process. The peroxidase · CN band at 538 $m\mu$ is clearly starting in 10^{-7} *M* cyanide

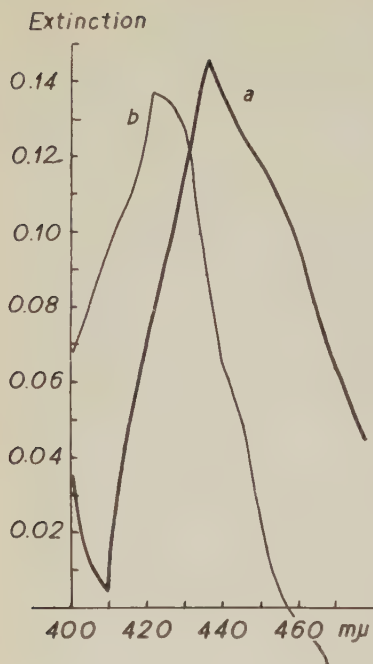


Figure 6. Difference spectrum ϵ peroxidase \cdot CN— ϵ peroxidase in living wheat roots (a), compared with difference spectrum ϵ anaerobic— ϵ anaerobic (b). The latter spectrum represents the completely reduced bands of the cytochromes, the former the peroxidase+partially reduced bands of the cytochromes a, c, b, and dh.

and is completely developed in a 10^{-4} M solution. In 10^{-5} M HCN the bands appear in about 15 seconds and are completely developed in 2—3 minutes (figure 7). If the growth of the difference band (cf. table 2) is measured at different wave-lengths it is noticed that the extinction at 540 mμ develops more rapidly than at 550 mμ (see figure 7). This is particularly obvious, if the difference is calculated between the observed extinction values at 550 and the calculated peroxidase \cdot CN values for this wave-length. This difference corresponds to the proceeding of the reduction of cytochrome c under the influence of the inhibited cytochrome oxidase. The reduction of cytochrome c proceeds considerably slower than the development of the peroxidase \cdot CN compound, its half-time (see 5) amounting to 120 seconds against 68 seconds for the peroxidase \cdot CN compound. The extremely rapid response of living roots to cyanide illustrates the rapid diffusion of the molecules of HCN through the tissue. The interesting difference in the velocity of response of peroxidase and cytochrome c is probably explained by the fact that the reaction between peroxidase and cyanide is a direct chemical process whereas the increased reduction of cytochrome c is the result of a displacement in the steady states of the total cytochrome system, initiated by the cyanide inhibition of cytochrome oxidase. In the adjustment of the steady states of the single cytochromes also the $\text{ATP} \rightleftharpoons \text{ADP}$ equilibrium, which is

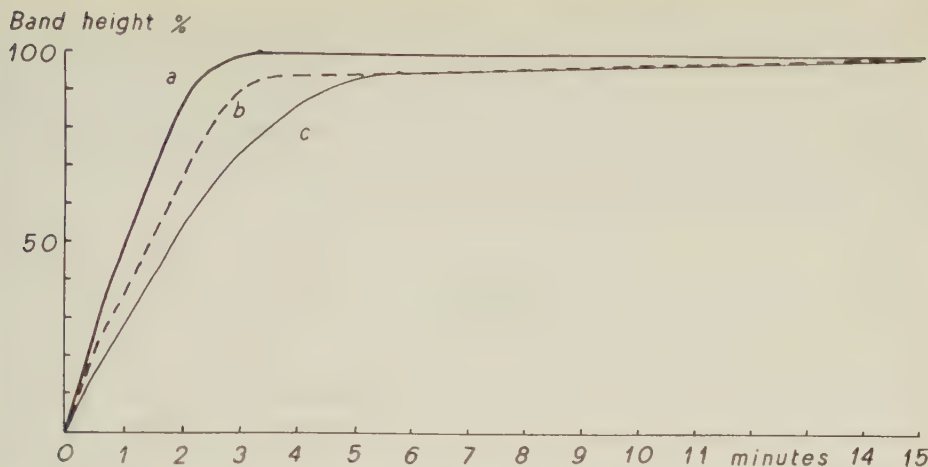


Figure 7. The time course of increasing formation of the peroxidase-CN complex (curve *a*), measured at 540 m μ , and the combination with the partial reduction of cytochrome *c*, measured at 550 m μ (curve *b*). Curve *c* represents the calculated net reduction of cytochrome *c*. See the text.

coupled to the electron transference $dh \rightleftharpoons b$, comes into play and retards the speed of reduction of cytochromes *b* and *c* (cf. 7).

The behaviour of the cyanide band in green does not support the idea of a series of CN-compounds of peroxidase, in analogy with the observed three complexes with H_2O_2 (1), but the possibility cannot yet be excluded that cyanide may be attached both to the prosthetic group (as a compound between trivalent iron and CN) and to some point of the apoenzyme and that the bands in violet and green are related to different groups in the haemin and thus able of partly independent variation, a possibility already touched upon in the literature (2). An observation of the behaviour of the cyanide band in the γ -region of the spectrum at a gradually increased concentration of HCN shows that an elevation at 428 m μ , corresponding to the difference band of peroxidase-CN, appears at low concentrations, but disappears at higher concentrations simultaneously with the displacement of the peak to 436—440 m μ . This comparatively slow displacement of the band cannot be attributed to the formation of a CN complex with cytochrome oxidase (4), because this is formed instantaneously. Besides of the possibilities that 1) wheat peroxidase is not quite identical with horse radish peroxidase, 2) that it is slightly modified under the influence of cyanide or, 3) that a second unknown compound develops a band in the region 440—450 m μ a further possibility ought to be considered, namely the slow formation of a cyanide complex with cytochrome *dh*. Such a compound would probably give a band in green.

too. A certain elevation of the cyanide spectrum at 580—585 m μ is sometimes observed and could hypothetically be matched with a γ -band at 435—440 m μ . The reoxidation of cytochrome *dh*, when going from anaerobic cyanide to aerobic cyanide, however, is not in accord with the idea of a CN complex formed with this compound. For a final decision between the mentioned possibilities more experimental facts are needed.

Summary

The spectrum of the cyanide and fluoride complexes with peroxidase from horse radish was compared with corresponding spectra from living tissues. Quantitative determinations of the apparent volume concentration of peroxidase can be made from the bands in the green and red parts of the spectrum. Wheat roots contain nearly as much peroxidase as horse radish. The coincidence with bands of cytochromes is discussed.

The work has been supported by the Research Councils of Science and of Agriculture.

References

1. Keilin, D. & Hartree, E. F.: Purification of horse-radish peroxidase and comparison of its properties with those of catalase and methaemoglobin. — *Biochem. Journ.* 49: 88. 1951.
2. Lemberg, R. & Legge, J. W.: Hematin compounds and bile pigments. — 1949.
3. Lundegårdh, H.: Spectroscopic evidence of the participation of the cytochrome-cytochromeoxidase system in the active transport of salts. — *Arkiv f. Kemi* 3: 69. 1951.
4. — Properties of the cytochrome system of wheat roots. — *Arkiv f. Kemi* 5: 97. 1952.
5. — Reaction kinetics of the cytochrome system. — 171: 521. 1953.
6. — A new cytochrome in living roots. — *Nature* 173: 939. 1954.
7. — On the oxidation of cytochrome *f* by light. — *Physiol. Plant.* 7: 375. 1954.
8. — Enzyme systems conducting the aerobic respiration of roots of wheat and rye. — *Arkiv f. Kemi* 7: 451. 1954.

On Partial Oxidation of the Cytochrome System in the Presence of Cyanide

By

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Introduction

The cytochrome system of the roots of young plants of wheat is provided with similar prosthetic groups as that of animal tissues, yeast, and certain bacteria, viz. is composed by cytochromes of the »families» *a*, *b*, and *c*. In addition to that wheat roots contain a fourth cytochrome, *dh* (6), which is characterized by an α -band at 571 m μ and a β -band at ca. 540 m μ . This cytochrome is under anaerobical conditions considerably more slowly reduced than cytochrome *b*, but it is rapidly reoxidized in the presence of oxygen. Its behaviour under the influence of a number of inhibitors reveals its properties as a member of the complete cytochrome system. Its prosthetic group is related to the haemin of cytochrome *b* and peroxidase (6, 7). A reversible transference of electrons is obviously going on between the cytochromes *b* and *dh*. The fact that no trace of a cytochrome *dh* has been found in heart muscle preparations, which are characterized by a high activity of succinodehydrogenase, seemingly speaks against its identity with this enzyme. It must be emphasized, however, that cytochrome *dh* remains highly oxidized unless the last traces of oxygen are consumed. Because the oxidized enzyme has no marked α - or β -bands its presence can be easily overlooked.

The cytochromes *a*, *c*, and *b* are rapidly and completely reduced if the aerated medium surrounding the roots is exchanged for an O₂-free medium (distilled water, phosphate buffer, or a solution of a neutral salt). The system remains intact even if the anaerobiosis is extended to 24 hours. It can, how-

ever, sometimes be observed that the reoxidation, which normally takes only few seconds, stops at a lower level of oxidation than before the anaerobiosis. The lag is more pronounced regarding the cytochromes *b* and *dh* than in respect of cytochrome *c* (and probably *a*) and may be interpreted as a retardation of the electron transference from *b* to *c*. This point is in fact one of the more sensitive in the total system, because a number of inhibitors are slowing down the electron transference between *b* and *c*. It is unlikely to assume that the action of the dehydrogenase is intensified by anaerobiosis. There are, on the other hand, no facts indicating a slackening of this enzyme in the absence of oxygen (cf. 5). The excess of reduction of the cytochromes *b* and *dh* after a period of anaerobiosis can amount to 20—40 per cent above the level held at the start in aerated solution.

Cyanide combines with cytochrome oxidase and hereby instantaneously inhibits 50 to 75 per cent of the total aerobic respiration of wheat roots (3). From observed differences between the absorption spectra recorded under complete anaerobiosis and after treatment with cyanide it was tentatively concluded (5) that also the cytochromes *dh* and possibly *b* are to a certain extent combined with CN. From biochemical preparations of cytochrome *c* and cytochrome *b* it is known that these enzymes only very slowly, if at all, combine with CN. The high percentage of peroxidase in the wheat roots (7) and the spectral behaviour of the peroxidase·CN complex introduces troublesome coincidences in the spectral picture. Since now the participation of peroxidase has been more closely studied (7) the problem of the effect of cyanide on the cytochrome system was reinvestigated.

Methods

The unbranched ca. 60 mm. long ends of the roots of 2—3 weeks old seedlings — grown in photothermostats at 25 °C. — were cut in lengths of 20 mm. The pieces were oriented parallelly in bundles which were tightly introduced in 40 mm. long quartz tubes of 16 mm. diameter. The tubes were closed by rubber stoppers provided with glass tubes for inlet and outlet of the streaming medium. This recipient was fastened on the object holder of the author's recording spectrophotometer (4). A layer of 12 strips of filter paper (Munktell no. 3) served as reference substance. The light absorption — sample and reference substance automatically shifted in 3.2 seconds — was recorded in spectral intervals of 2 mμ at a slit opening of 0.03 mm., corresponding to a band width of about 1 mμ in the α -region. At the recording of the absorption spectrum of the roots under aerobic conditions the medium flows through the recipient during the operation of the spectrophotometer. Anaerobiosis was brought about either by changing the solution from aerated to O₂ free, or by stopping the flow of the solution or by both means. As will be shown elsewhere there is a difference between these two ways of carrying through anaerobiosis, because in a flowing solution the carbon dioxide produced from the

anaerobic glycolysis is removed from the roots, whereas it accumulates at stopped flow. Carbon dioxide attacks the sensitive point of the system, mentioned above, viz. the electron transference between the cytochromes *b* and *c*, and may well be the chief cause of the lasting partial reduction of the cytochromes *b* and *dh* after prolonged anaerobiosis at stopped flow of the medium.

The basic medium was throughout 0.02 *M* phosphate buffer, pH 5.7. As KCN was never added to more than 0.001 *M* undue variations in the pH value were thus avoided.

The roots contain 100—200 per cent more peroxidase than cytochrome. For an accurate determination of the steady states of the cytochromes in the presence of cyanide the coincidence with peroxidase · CN must be eliminated. This can be done by calculation of the difference spectrum ϵ peroxidase · CN — ϵ peroxidase (7) and subtraction of it from the observed cyanide difference spectrum of the roots. The spectrum of peroxidase · CN appears in identical intensity both in aerated and O₂-free cyanide. The presence of cyanide does not interfere with the determination of the height of the reduced bands under anaerobiosis.

Reduction in the presence of aerobic cyanide

For these experiments four separate spectral recordings have to be made, namely of roots in (1) aerated phosphate buffer, (2) O₂-free phosphate buffer, (3) aerated phosphate + cyanide, and (4) O₂-free phosphate + cyanide. In some experiments also the reoxidation from (2) to (1), or from (4) to (3), was recorded.

From $\log \frac{\text{no. } 2}{\text{no. } 1}$ the relative molar concentration of the cytochromes can be calculated (cf. 4), from $\log \frac{\text{no. } 4}{\text{no. } 2}$ the concentration of peroxidase (cf. 7), and from $\log \frac{\text{no. } 3}{\text{no. } 1}$ the combined effects of the cyanide on peroxidase and cytochromes. For the calculation of the quantitative spectrum of peroxidase · CN the difference in extinction between the wave-lengths 540 and 525 m μ may be used. A useful correction is attained by determination of the pyridine haemochromogen (see 7).

The cyanide spectrum of O₂-free roots reflects the calculated difference curve of peroxidase · CN (figure 1), but a subsequent anaerobic oxidation of the cytochromes *b* and *dh* may cause deviations (see below). In aerated cyanide the region 545—580 m μ is higher than the calculated band of peroxidase · CN. In these cases (see figures 2 and 3) one peak is invariably found at ca. 550 m μ , corresponding to a partly reduced cytochrome of the *c* family, and a second peak or elevation at 563 m μ , corresponding to cytochrome *b*. The observed heights of the band at 550, 563, and 571 m μ minus the calculated heights of peroxidase · CN at the same points give the relative

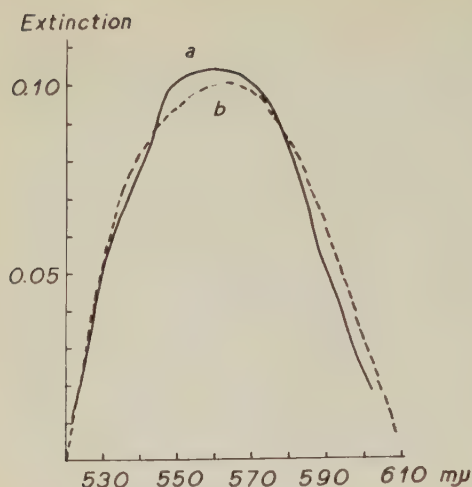


Figure 1. *Difference spectra of a 16 mm. thick bundle of wheat roots. Curve a: ϵ anaerobic cyanide— ϵ anaerobic phosphate, b: calculated curve of ϵ peroxidase · CN— ϵ peroxidase.*

reduction of the corresponding cytochromes. If the resulting figures are compared with the values for total extinction under complete anaerobiosis the percentual reduction in the presence of cyanide may be calculated (table 1).

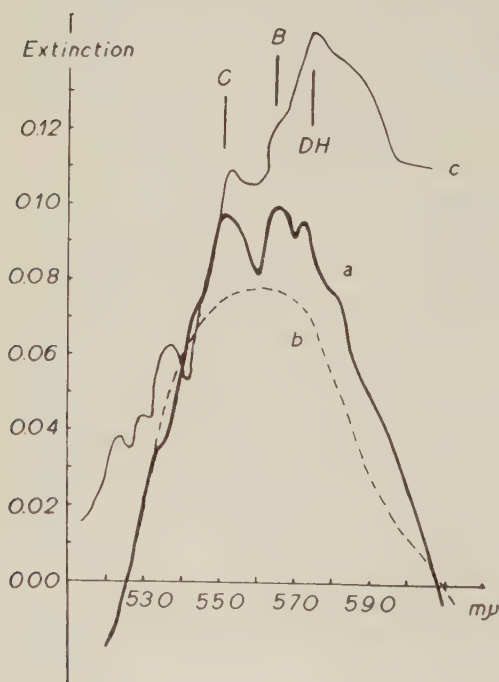


Figure 2. *Difference spectra of wheat roots. Curve a: ϵ aerobic cyanide— ϵ aerobic phosphate, b: calculated difference spectrum of peroxidase · CN, c: anaerobic phosphate— ϵ aerobic phosphate.*

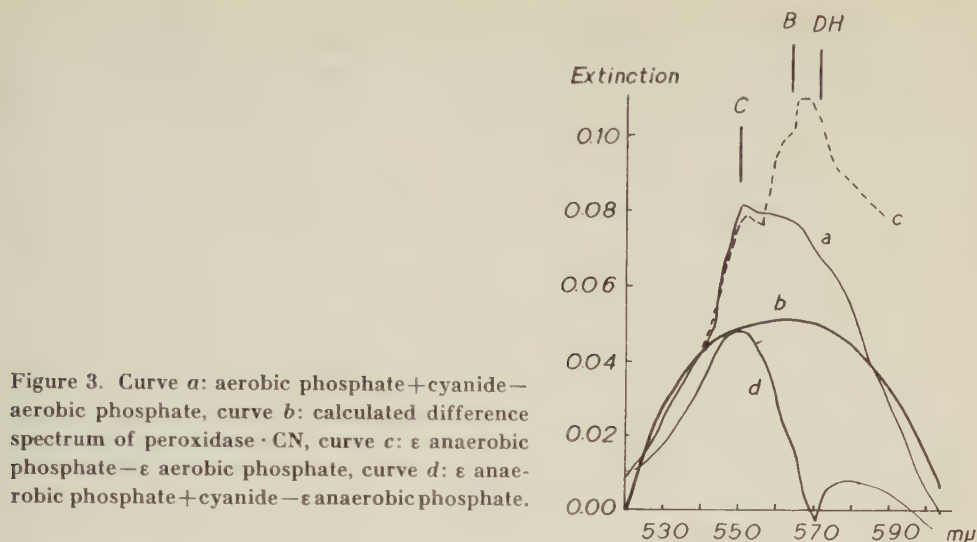


Figure 3. Curve *a*: aerobic phosphate+cyanide—
aerobic phosphate, curve *b*: calculated difference
spectrum of peroxidase · CN, curve *c*: ϵ anaerobic
phosphate— ϵ aerobic phosphate, curve *d*: ϵ anaerobic
phosphate+cyanide— ϵ anaerobic phosphate.

The bottom level of oxidation used for the calculations in table 1 corresponds to 80 per cent oxidation (see 4). The complete oxidation state can only be attained after treatment with substances as fluoride or malonate which in our case would have severely disturbed the experimental procedure. A correction of the values for total oxidation would not have altered the chief result, viz. the fact that the cytochromes *c*, *b*, and *dh* are only partially reduced in the presence of aerated cyanide and that the group *b-dh* is considerably less reduced than cytochrome *c*.

A close inspection of the cyanide spectrum of the roots does not indicate the formation of CN compounds of the cytochromes *c*, *b*, and *dh*. The slight elevation occasionally observed at about 580—585 $m\mu$, which was earlier believed to indicate a CN complex of cytochrome *dh*, is probably caused by the presence of still another haemin compound, but this question must be left open for the present. It may be mentioned, however, that peroxidase · CN gives a slight elevation at about 590 $m\mu$.

The partial reduction in aerobic cyanide is probably caused by the inactivation of the cytochrome oxidase as a consequence of which it cannot act as an acceptor of electrons from cytochrome *c*. The fact that the latter nevertheless remains oxidized to about 30 per cent can be attributed to one of the following two alternatives: (1) a retardation of the supply of electrons from the dehydrogenase via the cytochromes *b*, and *dh*; (2) the activity of an oxidase insensitive to cyanide and linked to the rest of the cytochrome system, including *c*, *b*, and *dh*. The alternative (1) is less probable because it is known that succinodehydrogenase is insensitive to cyanide, and because

Table 1. *Percental reduction of the cytochromes in the presence of cyanide under aerobic conditions.* The reduction is calculated from the normal predominantly oxidized state of the cytochromes taken as zero (see the text). Ten experiments.

Cytochrome <i>c</i>	Cytochrome <i>b</i>	Cytochrome <i>dh</i>
64	44	44
64	40	39
64	53	37
52	21	13
33	28	24
62	25	21
80	58	57
88	61	68
47	16	15
86	57	68
M: 64.0	40.3	37.6

even a heavily retarded transference of electrons would comparatively soon result in a complete reduction of the total cytochrome system, if the cytochrome oxidase were put out of action. The experiments with HCN under anaerobical conditions (see below) give conclusive evidence of the continual activity of the dehydrogenase.

As to the alternative (2) attention is called to the fact that a certain autoxidation of cytochrome *b* was observed at an early date (2), and that in vitro solutions of reduced cytochrome *c* are invariably slowly autoxidized even at pH values near 7. It has, however, never before been suggested that the autoxidation of cytochrome *b* could possibly act as a substitute for cytochrome oxidase in the living cell.

Reduction in and reoxidation from anaerobic cyanide

If the roots are transferred from aerated to O₂-free cyanide the reduction of the cytochrome system sets fort from the level reached in aerobic cyanide and may, particularly regarding cytochrome *c*, approach 100 per cent (figures 4 and 5). The increase of the reduced bands may also be observed on the difference spectrum of the Soret region (figures 4 and 5). The interpretation of the partial oxidation of the cytochromes *c*, *b*, and *dh* in aerated cyanide as a steady state between continuously running processes of oxidation and reduction is corroborated by the rapid occurrence of the reoxidation of the system if the medium is changed from anaerobic to aerobic cyanide.

The velocity constant (see 5) of the reoxidation in cyanide is about the half of that obtained for the reoxidation of cytochrome *c* in a dilute salt

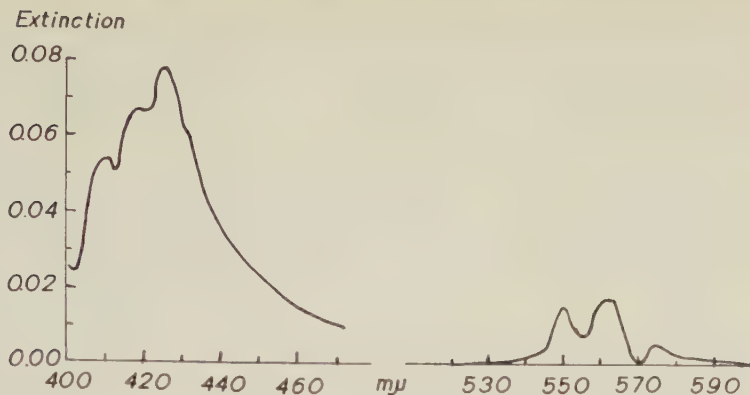


Figure 4. *Difference spectrum of wheat roots*, showing the additional reduction in going from aerobic to anaerobic cyanide+phosphate, or ϵ anaerobic cyanide+phosphate— ϵ aerobic cyanide+phosphate. The bands corresponding to the cytochromes *c* and *b* are dominating.

solution. The intensity of the process is hence sufficiently high to provide for the »ground respiration» of roots treated with cyanide (3), which only amounts to 25—50 per cent of the total aerobic respiration. The other fraction of the total respiration, the »anion respiration», is conducted by the cytochrome oxidase (oxidase I; see 4). The »oxidase II», which is responsible for the ground respiration, has not yet been identified. The pronounced insensitivity to cyanide speaks against its identity with a copper oxidase.

It was shown in a previous communication that the ground respiration varies synchronously with the quantity of flavoprotein and the possibility was discussed that oxidase II is an autoxidizable flavoprotein. The high turnover number (see 8), or 80 for FP as compared with 170 for cytochrome *b*, would indicate an exceptionally efficient flavoprotein. It may be tentatively suggested that the junction between flavoprotein and cytochrome *b* discussed in a previous communication (8) speeds up the oxidative qualities. A very close combination between flavoprotein and cytochrome was recently discovered by Appleby and Morton (1) as an enzyme identical with the lactodehydrogenase of bakers yeast. Because the investigations on wheat roots show that both flavoprotein and cytochrome are involved in the ground respiration the possibility of a close cooperation between these groups has to be seriously considered.

A close junction between flavoprotein and cytochrome *b* would explain why the processes linked to the stream of electrons from succinodehydrogenase to cytochrome *b* (see below) are so markedly slowed down at inactivation of the cytochrome oxidase. Flavoprotein is a link in the chain

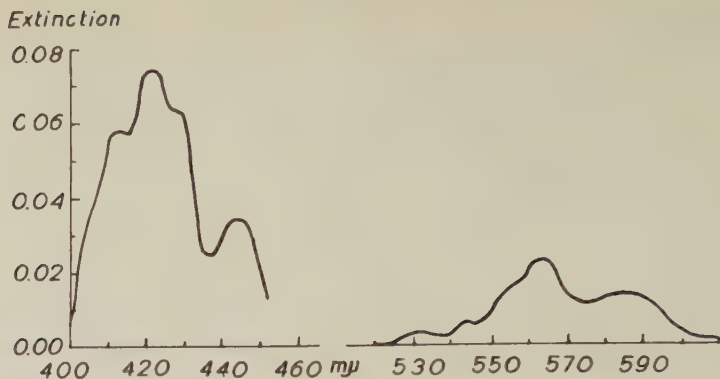


Figure 5. *Difference spectrum of wheat roots, showing the same as in fig. 4 but with a dominating reduction of cytochrome b.*

of processes conducting hydrogen and electrons from other points of the desmolysis of sugar than the equilibrium $\text{succinate} \rightleftharpoons \text{fumarate} + 2\text{H}$. The combination flavoprotein—cytochrome *b* is obviously capable of carrying through the oxidation of hydrogen collected from coenzyme I and similar sources but incapable of a simultaneous full oxidation of hydrogen supplied by succinodehydrogenase. For this task the much higher efficiency of the cytochrome oxidase is required. The key position of cytochrome *b* as a collector of electrons from two lines is probably the reason why this cytochrome (and *dh*) is quantitatively so dominating in the plant tissues.

The time-course of oxidation and reduction of cytochromes *c* and *b*

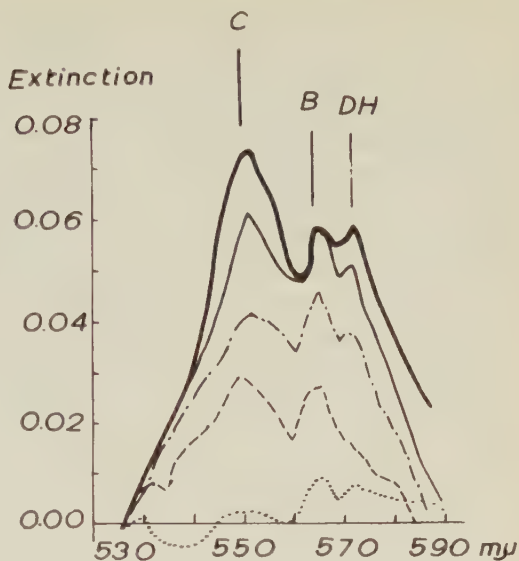
Observations of the time-course of reduction in aerobic 10^{-5} *M* cyanide (figure 6) show that cytochrome *b* is more rapidly reduced than cytochrome *c* but on the other hand stops at a higher level of oxidation than the latter. The half-times (*ht*; see 5) before reaching the steady states (see figure 6) are:

<i>c</i>	<i>b</i>	<i>dh</i>
200 sec.	36 sec.	70 sec.

The slow response of cytochrome *c* stands in remarkable contrast to its rapid reduction without cyanide, if the supply of oxygen is cut off. As shown in a previous communication (4) there exists a reversal equilibrium between the cytochromes *c* and *b*, electrons being able to move also from *c* to *b* if the latter is separately oxidized. This may possibly be one of the reasons why the

Figure 6. Time-course of increasing reduction of the cytochromes *c*, *b*, and *dh*, and of increasing formation of the peroxidase · CN complex in 10^{-5} m. cyanide + phosphate (streaming, aerated solution). Calculated as difference spectra.

..... 0—53 sec.
 ----- 53—106 sec.
 - - - - - 106—159 sec.
 ————— 159—212 sec.
 ————— 15 minutes.



response of cytochrome *c* is slowed down, because the oxidation in aerobic cyanide undoubtedly proceeds via cytochrome *b* instead of cytochrome oxidase. The speed of reduction of cytochrome *c* under anaerobiosis is clearly shown to depend on the relative molar concentrations $\frac{\text{cyt. } b}{\text{cyt. } c}$. At a high value of this relation cytochrome *c* is reduced at a higher speed than at a low value. The power of autooxidation of cytochrome *b* obviously acts in the same way as a reduced total quantity, viz. in direction of a decrease of its function as a donor of electrons to cytochrome *c*.

On anaerobic reoxidation of the cytochromes *b* and *dh*

At a prolonged stay in O_2 -free cyanide the reduction of the cytochromes *b* and *dh* decreases, as shown by a lowering of the bands (figures 1, 7). The anaerobic oxidation amounts to about 50 per cent. Cytochrome *c* is much less affected, only up to 10—15 per cent. The observed effect cannot be confounded with the formation of a cyanide complex with *b* and *dh*, because such complexes would impede the observed rapid reoxidation in the presence of cyanide. Also the fact that no lowering of the reduced bands appears in aerobic cyanide decidedly speaks against the formation of CN complexes. The decrease of the reduced bands can only be interpreted as an anaerobic oxidation.

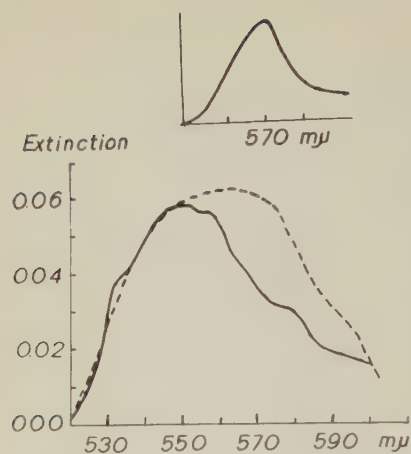
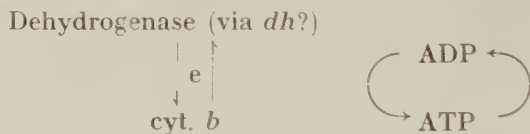


Figure 7. *Difference spectra of wheat roots, showing the anaerobic reoxidation of predominately cytochrome dh and to some extent b in the presence of cyanide. Lower curve: — ϵ anaerobic cyanide + phosphate — ϵ anaerobic phosphate; - - - - calculated difference spectrum of peroxidase \cdot CN. Upper curve: the difference between the two curves, showing the calculated anaerobic oxidation of dh (+ b).*

It was found in this laboratory that the cytochrome system of wheat roots is coupled to the formation of ATP or some similar \sim ph product. The experiments, which will be shown in a separate publication, support the assumption of a reversible equilibrium $ADP \rightleftharpoons ATP$ conducted by the electron transference between succinodehydrogenase and cytochrome b , possibly via cytochrome dh , according to the following scheme



If the on-reaction, viz. the stream of electrons from the dehydrogenase, stops owing to an absence of oxygen no ATP is formed, but the off-reaction, viz. the decomposition of ATP to ADP, may still proceed. The stream of electrons is now reversed, as a consequence of which cytochrome b is oxidized and in its turn oxidizes cytochrome dh . ATP acts as an internal oxidizing agent in the system and it may consequently be able to maintain a certain anaerobic oxidation of metabolites via the flavoprotein system. A similar reversed oxidation of cytochrome b is observed under the influence of fumarate or malonate (4). Because an anaerobic reoxidation of cytochrome b is not observed in the absence of cyanide this agent probably interferes with the phosphorylation in such a way that the retarding effect is focussed on the on-reaction. This effect of cyanide is rather specific, because it conveys a pronounced oxidation of cytochrome dh . Fumarate also oxidizes cytochrome b , but has the reversed effect on cytochrome dh .

Summary

A reinvestigation of the effects of cyanide on the cytochrome system of wheat roots showed that under normal pH conditions of the living tissue CN complexes are not formed with the cytochromes *c*, *b*, and *dh*. The observed effects are confined to the inactivation of the cytochrome oxidase and the formation of a peroxidase · CN complex. In an aerated solution of cyanide the cytochromes *c*, *b*, and *dh* are only partially reduced, cytochrome *c* to about 60, the two others to about 40 per cent of the space between predominant oxidation in an aerated salt solution and complete reduction under anaerobiosis. The succinodehydrogenase is not inhibited by cyanide, as a consequence of which anaerobically complete reduction is attained also in the presence of cyanide. The velocity of reoxidation in the presence of cyanide amounts to about half of the velocity of the intact cytochrome system. The forthcoming experimental evidence supports the idea that cytochrome *b* acts as a substitute for cytochrome oxidase but at considerably reduced power, only sufficient for carrying on an oxidation via flavoprotein, but incapable of oxidizing noteworthy quantities of succinate. The possibility was discussed that a close junction exists between cytochrome *b* and flavoprotein and that this complex constitutes the enzyme of ground respiration, whereas the enzyme of anion respiration is cytochrome oxidase. Besides of its effect on cytochrome oxidase and peroxidase cyanide also probably affects the ~ph balance, thus promoting an anaerobic oxidation of the cytochromes *b* and *dh*.

The work has been supported by the Research Councils of Science and of Agriculture.

References

1. Appleby, C. A. & Morton, R. K.: Crystalline cytochrome b_2 and lactic dehydrogenase of yeast. — *Nature* 173: 749. 1954.
2. Keilin, D. & Hartree, E. F.: Cytochrome and cytochrome oxidase. — *Proc. Roy. Soc., B* 127: 167. 1939.
3. Lundegårdh, H.: The translocation of salts and water through wheat roots. — *Physiol. Plant.* 3: 103. 1950.
4. — Properties of the cytochrome system of wheat roots. — *Ark. f. kemi* 5: 97. 1952.
5. — Reaction kinetics of the cytochrome system. — *Nature* 171: 521. 1953.
6. — A new cytochrome in living roots. — *Nature* 173: 939. 1954.
7. — Spectrophotometrical determination of peroxidase in living roots. — *Physiol. Plant.* 8: 000. 1955.
8. — Enzyme systems conducting the aerobic respiration of roots of wheat and rye. — *Arkiv f. Kemi* 7: 451. 1954.

Influence of pH on the Respiration in *Chlorella pyrenoidosa*

By

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Very little has been published about the influence of pH on the rate of respiration in aquatic plants. Only some scattered remarks are found in articles describing primarily experiments of another kind. Emerson and Green (1938) stated that the respiration in *Chlorella* is about 30 per cent higher at pH 4.6 than at pH 7.0 or 8.9. Steemann Nielsen (1947) found, however, that the rate of respiration in *Myriophyllum spicatum* is about 20 per cent lower at pH 4.5 than at pH 9.3.

The influence of pH on the rate of photosynthesis in aquatic plants has been worked out to a much higher degree, e.g. Emerson and Green (1938). Steemann Nielsen (1947) and Österlind (1949). The first mentioned workers thus made an important contribution regarding the influence of pH in *Chlorella*. They found a constant photosynthetic rate in the pH-range 4.6—8.9 when both CO₂ and light were saturated. Under certain circumstances a decreasing effect of low pH on the photosynthetic rate in saturated light in *Chlorella* may be found (Steemann Nielsen in press). Normally, however, the rate is independent of pH.

During some *Chlorella* experiments where pH had to be varied it was of importance to know the exact behavior of the species. It was thus necessary to make a detailed investigation concerning the influence of pH on the rate of respiration. It soon became apparent, however, that a clear picture of this influence was extremely difficult to demonstrate. The three representative curves in Figure 1 show clearly how the influence of varying pH may differ from series to series.

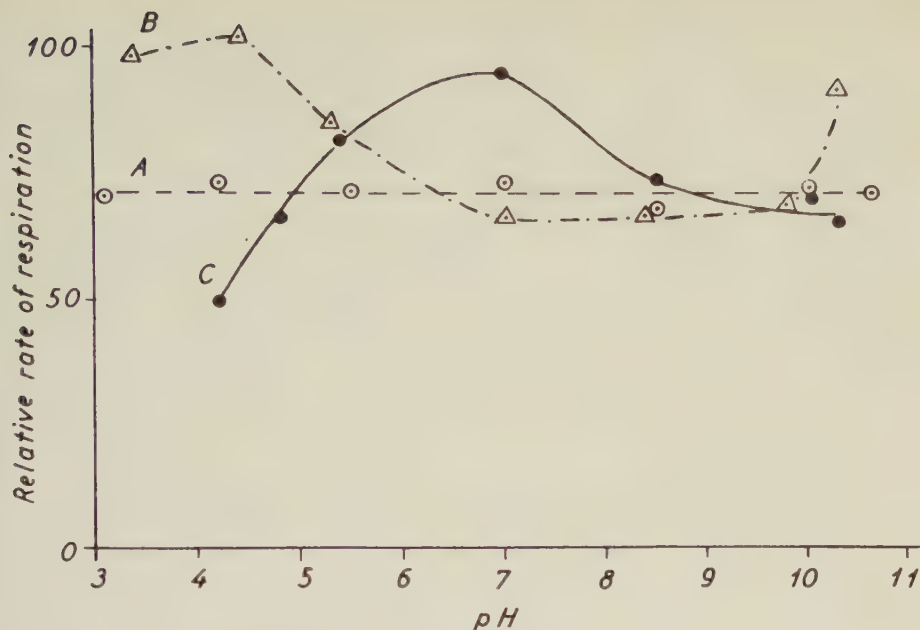


Figure 1. Rate of respiration in *Chlorella* as a function of pH. 3 different series.

The rate of respiration was measured by determining the oxygen uptake by the algae by means of the Winkler-method (see Steemann Nielsen 1953). 15 ml. bottles with glass stoppers were used. All single determinations were made in duplicate. The algae had been cultivated in Drechsel gas-washing bottles according to the description in the above mentioned paper. The cultures were aerated by a mixture of 5 per cent CO_2 in air.

In the experiments carbonate buffers were used consisting of mixtures of 0.03 N KHCO_3 and K_2CO_3 with addition of small concentrations of CaCl_2 (35 mg/l), K_2SO_4 (10 mg/l) and NaCl (5 mg/l). At low pH, 5 per cent free CO_2 was used for buffering. As only dilute suspensions of *Chlorella* were employed the buffering action was adequate. In some special experiments phosphate buffers were used. All experiments were made at 21°C .

The duration of the experiments shown in Figure 1 was about 5 hours in series A, about 4 hours in series B and about $2\frac{1}{2}$ hours in series C. Because of the low algal concentration used, experiments of rather long duration were necessary. The variation between the duplicates at any given pH in the different series was always small. In series A the rate of respiration is independent of pH. In series B there is a definite minimum in the pH range from 7 to nearly 10 showing a definite increase of the rate both at lower and at higher pH values. In series C on the other hand the maximum rate of respira-

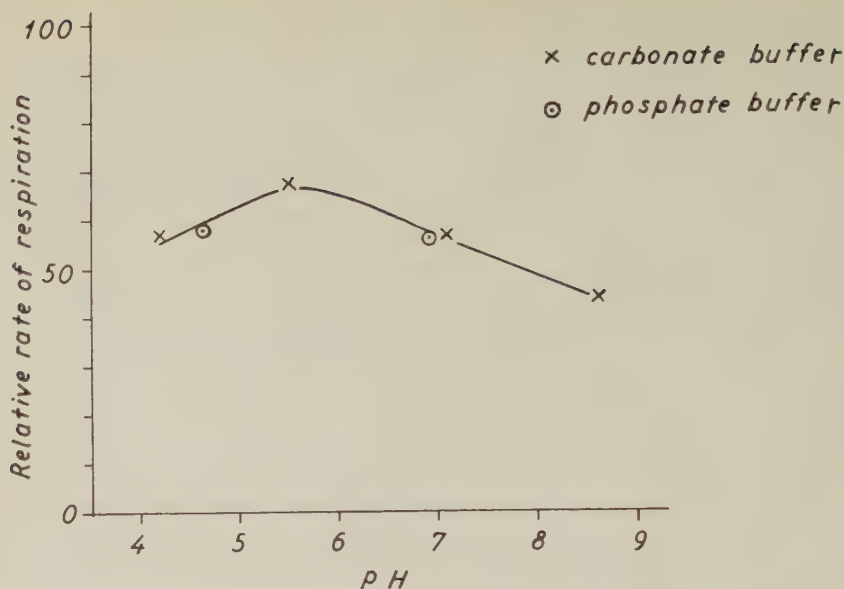


Figure 2. Rate of respiration in *Chlorella* as a function of pH. Carbonate or phosphate used for buffering.

tion was measured at pH 7 the curve declining both towards lower and higher pH values.

The differences between the three series of experiments were confusing. A program was therefore set up to investigate this peculiar behavior, and if possible, to obtain some understanding of its background.

The experiments shown in Figure 2 were made to investigate whether the method of buffering the media has any influence on the shape of the pH-dependence-curve. The results of the experiments where phosphate mixtures of 0.04 n KH_2PO_4 and 0.04 n KH_2PO_4 were used as a buffer, however, fit excellently into the curve drawn from the experiments where carbonate-carbon dioxide was used as a buffer. In a previous article (Steemann Nielsen 1953, p. 321) it was shown that variations of the carbon dioxide concentration between 0.4 and 5 per cent have no influence on the rate of respiration of *Chlorella* in the dark. The experiments illustrated in Figure 2 also demonstrate this fact, since very little CO_2 was present in the phosphate buffer. This does not prove definitely, however, that CO_2 in as high a concentration as 5 per cent never may influence the rate of dark respiration in *Chlorella*. As shown by Steemann Nielsen (paper in press) the narcotic effect of CO_2 has many aspects.

The pH of the cultures used for the experiments illustrated in Figure 1

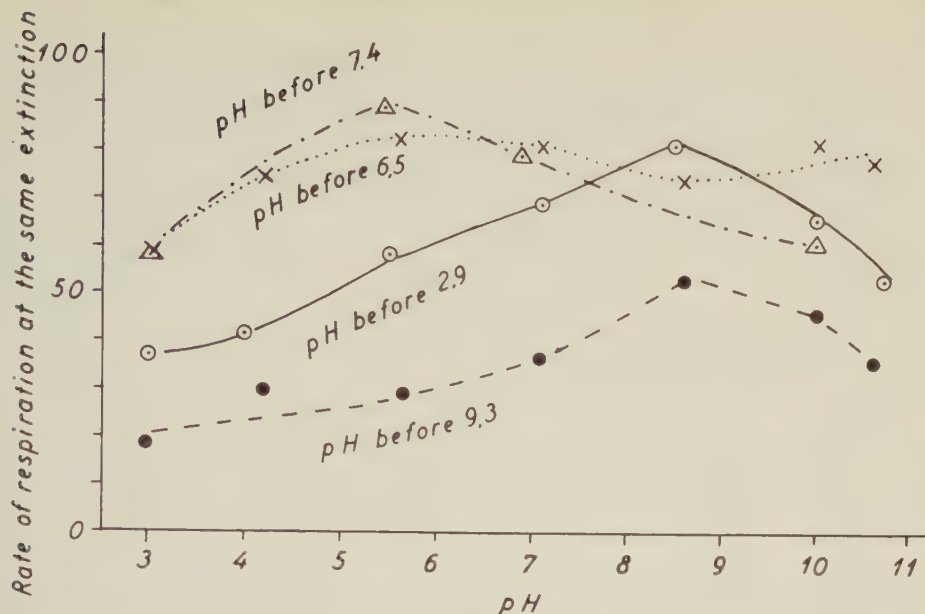


Figure 3. Rate of respiration in *Chlorella* as a function of pH. Cultures grown at different pH, used for the experiments.

was almost constant, ranging from an initial value of 4.6—5.0 to a final value of 6.5. However, in some other series (Fig. 3) the final pH of the cultures employed varied from 2.9 to 9.3. In these series, the culture having a final pH of 2.9 was 4 days old when used and contained NH_4^+ (0.75 g NH_4Cl per litre) for an N-source rather than NO_3^- . That having a final pH of 6.5 was grown in normal media and was used after 3 days. The culture with a final pH of 7.5 was started in normal media to which 1.5 g KHCO_3 per litre was added during the second day, and was used after 3 days of growth. The culture which had a final pH of 9.3 was grown in normal but in aerated media under natural illumination from a north window during March. This culture was used after 20 days, at which time it appeared to be perfectly healthy.

The rates of respiration in the series shown in Fig. 3 are arbitrary but given for the same density of algae as measured with an Eel colorimeter with a blue filter. The maximum rates of respiration were nearly the same in the different series with the exception of the series where a culture at pH 9.3 was employed. A reduction of about $\frac{1}{3}$ was found here, possibly because of the age of the cells used in this case.

The shapes of the curves illustrating the dependence of rate of respiration on pH appear to bear no relation to the pH of the culture employed. The two series where cultures showing the two extreme pH values (2.9 and 9.3)

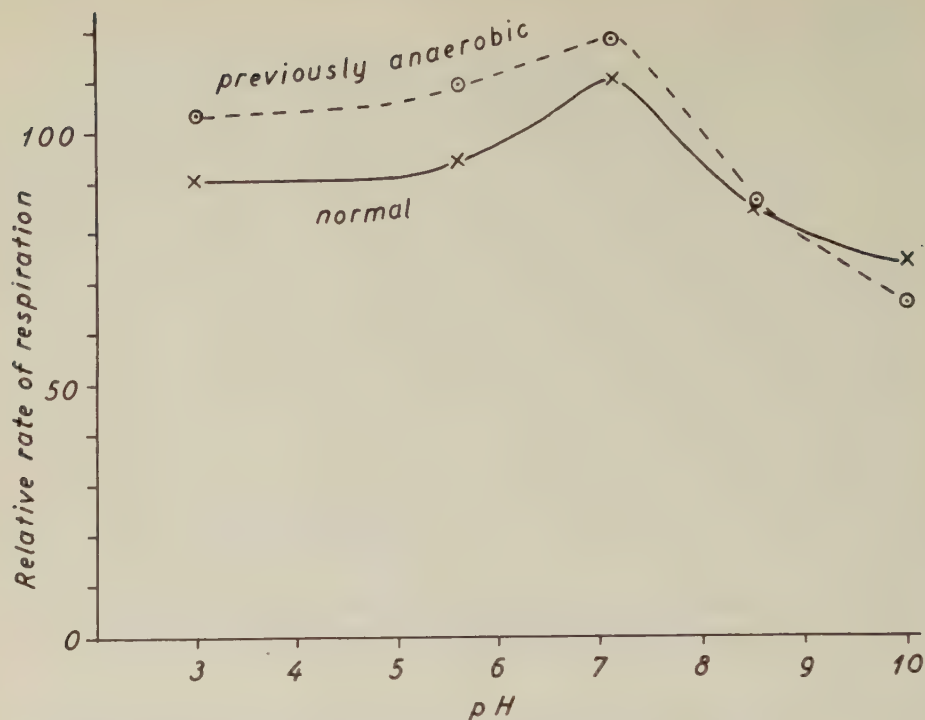


Figure 4. Rate of respiration in *Chlorella* as a function of pH. With or without a previous anaerobic treatment for one hour.

appear to be most similar. The maximum rate of respiration was in both series obtained at pH 8.5. In the two other series the maximum rate was found about pH 5.5. The duration of all these experiments was rather long, ranging from 4 to 6 hours.

Figure 4 shows some experiments made to investigate if anaerobic conditions previous to the experiments may have any influence on the shape of the pH-dependence-curve. As the algae were centrifugated before the start of an experiment anaerobic conditions could possibly be produced at the bottom of the centrifuge tube where the algae were packed together. The algae from a 3 days old culture (pH 6.9) were after centrifugation divided into two parts. The one part was used immediately for a series of experiments, the other consisting of packed cells in a minute bottle with glass stopper was left under anaerobic conditions for one hour. The shapes of the curves, however, are nearly identical.

Figures 5-7 illustrate experiments showing the influence of the time course on the shape of the pH-dependence-curves. In Figure 5 the full line

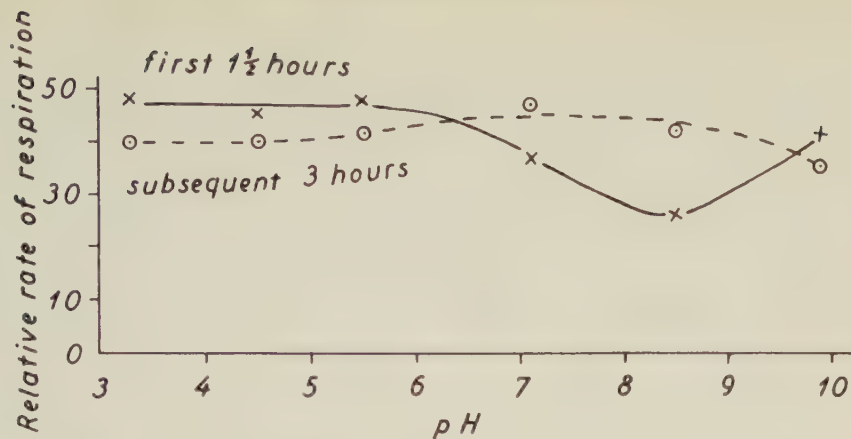


Figure 5. Rate of respiration in *Chlorella* as a function of pH and time.

shows the dependence on pH during the first 1 1/2 hours at the different pH values, the dashed line shows the dependence during the subsequent 3 hours. A real difference is to be seen. Whereas the maximum rate during the first 1 1/2 hours was found at pH 3—5 and the minimum rate at about pH 8, during the subsequent 3 hours the rates at pH 3—5 had decreased whereas

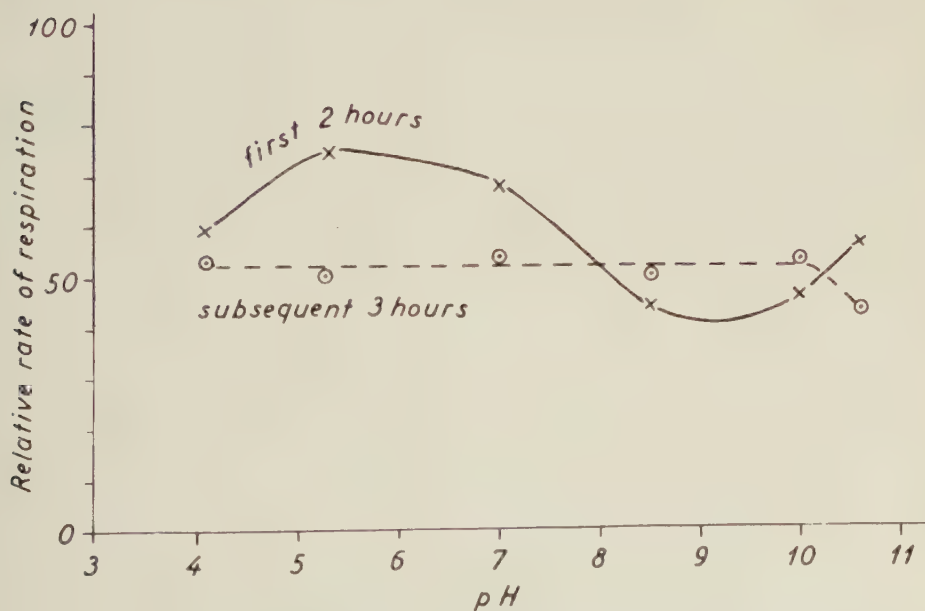


Figure 6. Rate of respiration in *Chlorella* as a function of pH and time.

the rates at pH 7 and particularly at pH 8 had increased so that the curve now nearly shows independence of the rate of respiration on pH with only a slight optimum at a pH about 8. Figure 6 illustrates a series, the result of which was rather like the series just mentioned above. After the first two hours the respiratory rates were identical in the pH range 4—10. Figure 7 finally shows a series in which the respiratory rates were measured at pH 4.5 and 8.3 for one hour periods during the four first hours of the treatment at the pH in question. At the start the rate was by far the highest at pH 4.5. During the first two hours the rate decreased at pH 4.5 but increased at pH 8.3. During the fourth hour the rates were identical at pH 8.3 and 4.5.

The time factor thus seems to be very important in influencing the shape of the curve illustrating the dependence of the respiratory rate in *Chlorella* on pH. It may, however, be supposed that the time factor possibly is not the only one responsible for the different shapes of the curves shown in this article. More investigations are absolutely needed. It seems, however, that the rate of respiration after a couple of hours where the pH dependence is very complicated becomes independent of the external pH.

Discussion

How does pH influence the rate of respiration in *Chlorella*? The rate of photosynthesis is as stated above ordinarily independent of the pH in the surrounding water at light saturation where the overall process is limited by enzymatic processes. No time course seems necessary for establishing this independency — unlike conditions for respiration. The protoplasm must thus in any case in light normally in some way or other be able at all times to maintain a rather constant pH independent of the pH of the surrounding medium or the enzymes involved in photosynthesis must be independent of pH in a very wide range which is rather unlikely.

It has been shown by Scott 1945 that pH has a marked influence on the mineral composition of *Chlorella*. At a high pH the amount of cations (Ca^{++} , Mg^{++} , K^+) in the cells increases. At low pH the amount of Ca^{++} and K^+ decreases. Some phosphate ions are lost at the same time. This is assumed by Scott to be due to the instability in an acid medium of certain phosphate containing compounds in the cell and to a decrease or an increase in the base-binding capacity of cellular constituents.

According to this the buffering action of the cell due to organic compounds of amphoteric nature — probably the proteins — is able to neutralize the invasion of bases or acids into the cells for some time at least.

In the long run, however, another mechanism must be assumed to be re-

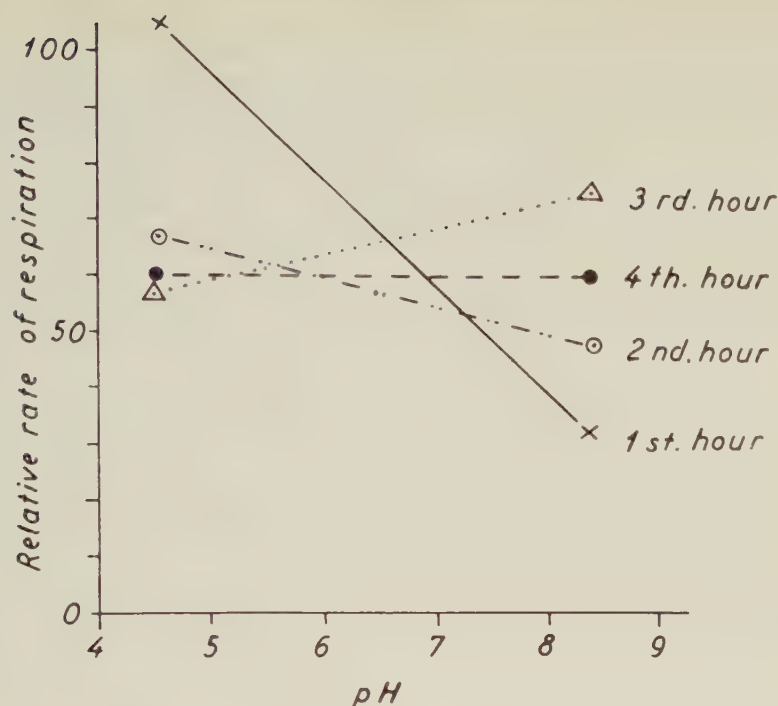


Figure 7. Rates of respiration in *Chlorella* at pH 4.5 and 8.3 as a function of time.

responsible for the prevention of any important change of the pH of the protoplasm. This mechanism works to prevent the invasion of OH^- or H^+ ions. After about 1—2 hours when the mechanism is finally established the rate of respiration is independent of pH. This seems to indicate that the mechanism in question — or another one — is able to re-establish the normal pH of the protoplasm if any change has taken place.

It is not possible at present to say anything definitely about the nature of the mechanism responsible for the exclusion of H^+ and OH^- ions. Since it is not momentarily functioning when the cells are transferred to a media having either higher or a lower pH, it is very important that the protoplasm to some extent is able to neutralize the invasion of H^+ and OH^- for some time.

According to the experiments illustrating the influence of the time factor — Figures 5—7 — the initial respiratory rates at a pH of about 8–9 are lower than the final one whereas the initial rates at low pH are higher. The behavior at a very high pH (about 10, see below) is preliminarily left out of consideration. It seems that the initial buffering by the mentioned com-

pounds of amphoteric nature is insufficient to keep the internal pH absolutely constant.

If the rates of the enzymatic reactions involved in respiration decrease when the internal pH increases the initial low rates of respiration in the pH range 8—9 are understandable. If on the other hand the optimum pH of the enzymatic reactions in question should be a little lower than normally found inside the cells the increase of the respiratory rate during the initial stay at a low pH can be explained as the result of a slight decrease of the internal pH. The lower initial rate of respiration at pH 4 compared with that at pH 5 according to the curve for the first two hours in Figure 6 would thus indicate that at an external pH of 5 the internal pH has just decreased to the optimum value whereas at an external pH of 4 where the invasion rate of H^+ ions is higher, the internal pH has decreased so much that it has become suboptimal.

In quite a number of the series where the influence of the time factor was not specially investigated the peak of the pH-dependence curve was found about pH 6—7, at which pH the algae had grown before. A typical example is e.g. found in series C, Figure 1. It is presumably to be expected that the invasion of H^+ and OH^- ions is relatively high in these series. The proportion between the maximum and the minimum rate observed is in any case high.

Most difficult to explain are the relatively high respiratory rates sometimes found at a very high pH (series B, Fig. 1 and the initial values, Fig. 5 and Fig. 6). It has perhaps something to do with the fact that *Chlorella pyrenoidosa* at a pH above 10 is close to the limit for survival. An emergency mechanism requiring energy is perhaps started.

As mentioned above, the rate of photosynthesis in *Chlorella* is occasionally but not normally dependent on pH. The immediate independence of photosynthesis on pH normally found is presumably an indication that the establishment of the mechanism responsible for the exclusion of H^+ and OH^- ions is accelerated in light. It is rather likely that the reason for the occasional behavior when the algae are dependent on pH is to be sought in conditions effecting a delaying of the establishment of the mechanism in question. The depression of the photosynthetic rate at a low pH can be counteracted by very high CO_2 concentrations (see Steemann Nielsen in press). Whether this also is the case in respiration under the same conditions has not yet been investigated.

In all an altering of the pH of the surrounding medium appears to give rise to several events in the *Chlorella* cells. The problem may seem rather complicated. It is, however, to be hoped that the present investigations may stimulate the interest in studying the influence of pH on algal cells.

Summary

The dependence of the respiratory rate of *Chlorella pyrenoidosa* on pH varies greatly in apparently identical series of experiments.

The pH effects is independent 1) on the method of buffering the media, 2) on aerobic or anaerobic conditions just before the experiments, and only to a slight and apparently unsystematic degree, 3) on the pH at which the algae have been cultivated.

The time factor on the other hand is important. After 1—2 hours treatment at the various pH the rate of respiration seems to become independent of pH.

The first effect of either a low or a high pH is apparently an invasion of either H^+ or OH^- ions into the cell. The proteins here being amphoteric substances are able to act as buffers at a certain extent for some time. A special mechanism is, however, soon started which prevents any further invasion of either H^+ or OH^- ions and reestablish the original pH.

A relatively low rate of respiration at the start of the exposure to a low or high pH may be caused by the decrease in the rate of some enzymatic processes involved in respiration, due to insufficient buffering of the protoplasm before the mechanism mentioned above is fully established.

A relatively high rate of respiration at the start of the exposure to a low pH may on the other hand be a consequence of an only slight decrease of the internal pH which perhaps normally is slightly on the alkaline side of the optimum for the processes in question.

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References

- Emerson, R. & Green, L.: Effect of hydrogen-ion concentration on *Chlorella* photosynthesis. *Plant Physiol.* 13: 157. 1938.
- Scott, G. J.: The influence of H-ion concentration on the mineral composition of *Chlorella pyrenoidosa*. *Journ. Cellular and Compar. Physiol.* 25: 37. 1945.
- Steemann Nielsen, E.: Photosynthesis of aquatic plants with special reference to the carbon-sources. *Dansk Botanisk Arkiv* 12, Nr. 8: 1. 1947.
- Carbon dioxide concentration, respiration during photosynthesis and maximum quantum yield of photosynthesis. *Phys. Plant.* 6: 316. 1953.
- Carbon dioxide as carbon source and narcotic in photosynthesis and growth of *Chlorella pyrenoidosa*. *Phys. Plant.* in press.
- Österlind, S.: Growth conditions of the alga *Scenedesmus quadricauda* with special reference to the inorganic carbon sources. *Symb. Botan Upsaliens.* X 3: 1. 1949.

Inhibition of Bacterial Sulphate-Reduction in Presence of Short Chain Fatty Acids

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Introduction

Hoppe-Seyler (1886) was the first to prove the occurrence of hydrogen sulphide in the course of methane fermentation of cellulose by impure enrichment cultures of anaerobic bacteria. He held that the sulphide arises through reduction of sulphate by methane *in statu nascendi*. Rubentschik (1928) showed that the above cultures contain vibrios belonging to the genus *Desulphovibrio* (*Microspira*). Further he demonstrated that the lower fatty acids (acetic and butyric acid) formed in anaerobic bacterial decomposition of cellulose may be utilized by these vibrios in reducing sulphate to hydrogen sulphide. Using freshly isolated pure cultures Baars (1930) was able to distinguish the two species *Desulphovibrio desulphuricans* and *Desulphovibrio rubentschickii* on basis of their power to utilize the lower fatty acids as hydrogen donators in transforming sulphate to sulphide. Strains of the former organism are incapable of attacking acetic, propionic and butyric acid, whereas in cultures of strains belonging to the latter species a complete oxidation of these compounds to carbon dioxide and water is brought about provided that sufficient amounts of sulphate are present. Consequently acetic, propionic and butyric acids are found as end-products of fermentation in cultures of *D. desulphuricans* supplied with the respective alcohols (ethanol,

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n-propanol and n-butanol) as donators of hydrogen, whereas in cultures of *D. rubentschickii* containing the same alcohols no accumulation of fatty acids takes place. According to Baars (1930) the last-mentioned species, however, includes strains capable of attacking propionic and butyric acid but incapable of utilizing acetic acid, this acid thus accumulating in cultures supplemented with higher alcohols and fatty acids (n-propanol, propionic acid, etc.) as hydrogen donators (*Vibrio rubentschickii* var. *anomalus*).

Baars (1930) studied the sulphide production by *D. desulphuricans* in lactate media compared to that in substrates containing a mixture of lactate with butyrate and acetate respectively. The amounts of hydrogen sulphide developed in the cultures after 7 days of incubation corresponded approximately to the quantities of lactate added. Baars (1930) does not mention anything about an inhibition of sulphate reduction in the lactate cultures supplemented with fatty acids as compared to those supplied with only lactic acid as a hydrogen donator and as a source of carbon. Apparently the strain of *D. desulphuricans* examined by Baars (1930) might not have shown either a marked increase in induction time or a striking decrease in rate of sulphate reduction in presence of lower fatty acids. Summarizing, we may according to the classical works of Rubentschik (1928) and Baars (1930) distinguish the following physiological types as far as the metabolism of lower fatty acids with simultaneous reduction of sulphate to sulphide is concerned:

Typical strains of *D. rubentschickii*: Complete oxidation of fatty acids to carbon dioxide and water.

Atypical strains of *D. rubentschickii* (var. *anomalus*): Incomplete oxidation of fatty acids to acetic acid, carbon dioxide and water. No decomposition of acetic acid.

Typical strains of *D. desulphuricans*: No utilization of fatty acids.

The findings described in the present paper prove the existence of strains of *D. desulphuricans* characterized not only by total inability to attack the lower fatty acids but even through a high sensitivity to the action of these acids, pronounced inhibition of growth and sulphate reduction being obtained in lactate media supplemented with acetate, propionate and n-butyrate respectively.

The strong toxic effect produced by the lower fatty acids upon the strain of *D. desulphuricans* dealt with in the present paper seems to be somewhat surprising in view of the fact that closely related organisms, as reviewed above, are capable of utilizing the same acids in their normal metabolism for reduction of sulphate. In this connection it may, however, be called to mind that the fatty acids in several cases have been found to exhibit considerable bacteriostatic and fungistatic properties of practical significance, formate («Amasil») being successfully used in preparation of silage and propionate being

considered a promising preservative for bread, cheese, fruits and vegetables. As to the proper literature we refer to Hoffman, Schweitzer and Dalby (1939), Eichholtz (1941), Richard and Heinzl (1942), Wolford and Andersen (1945), Heseltine (1952), Somm (1953 a and b), Wikén and Zobrist (1953). In some cases even the mechanism of the inhibitory action of short chain fatty acids is known. Thus King and Cheldelin (1948) have shown that propionate inhibits growth of strains of *Saccharomyces cerevisiae* and *Acetobacter suboxydans* probably by competing with β -alanine in cellular synthesis of pantothenic acid. Further, Hill (1952) has reported that propionate may inhibit growth of *Streptococcus faecalis* by combining with coenzyme A to propionyl CoA, thereby interfering with the formation of acetate from pyruvate. In this connection it is interesting to note that pantothenic acid is a part of the molecule of this coenzyme (Lipmann et al. 1950, Gregory et al. 1952, Lipmann 1953).

Materials and methods

The organism examined was the Zürich strain of *D. desulphuricans* (cf. Wikén and Ghose 1954).

The basal substrate employed for the tests was the synthetic lactate-sulphate medium of Starkey (1938), modified as to contain 27—41 mM of lactic acid (3.0—4.5 g. of sodium lactate) and 27—35 mM of sulphate (0.3—0.4 g. of Mohr's salt + 2.0 g. of magnesium sulphate + 5.5—8.5 g. of sodium sulphate) per 1000 ml. in addition to ammonium chloride, dipotassium phosphate and calcium chloride (cf. Wikén and Ghose 1954). The concentrations of fatty acids tested were 11.6—89.2 mM of acetic acid, 7.4—97.3 mM of propionic acid and 10.6—100.3 mM of n-butyric acid per 1000 ml. of substrate. The initial pH was adjusted to 7.0—7.5. The solution of Mohr's salt (20 per cent by weight) was sterilized separately by Seitz-filtering and added aseptically after autoclaving and cooling the media under a current of oxygen-free nitrogen.

Jena glass bottles (*Jena Geräteglas 20*), each containing 5 litres of liquid medium, were used as culture vessels. They were sealed anaerobically as described by Wikén and Ghose (1954, Figure 1).

The inoculum used per 1000 ml. of substrate amounted to 5 ml. of a liquid culture grown anaerobically (soda-pyrogallol seal) in the above lactate-sulphate medium (basal substrate without fatty acids) for 48 hrs. in Experiments 1 and 2 and for 20 hrs. in Experiment 3.

All cultures (stock cultures, subcultures used for inoculation, test cultures) were incubated in the dark at a temperature of 37° C.

The methods employed in assaying the rate of metabolism in presence of different amounts of fatty acids were the same as described previously (Wikén and Ghose 1954). Thus lactic acid was determined according to Friedemann, Cottonio and Shaffer (1927). The fatty acids were identified and estimated by means of Duclaux distillation and silica gel partition chromatography according to Elsdén (1946), after steam

distillation and oxidation by the method of Friedemann (1938). Sulphate was determined gravimetrically (Treadwell 1930, Kolthoff and Sandell 1947) and sulphide by iodometric titration (Starkey 1938).

Experiments and results

This work describes the results obtained in typical experiments with proliferating cells of the Zürich strain of *D. desulphuricans* in lactate-sulphate medium containing *single* fatty acids in different concentrations. In the last experiment reported here the effect produced by *n*-butyrate is compared to that obtained with the same inoculum in parallel cultures supplied with acetate and propionate respectively.

Experiment 1

The rate of metabolism observed in the lactate-sulphate medium without addition of fatty acid was compared to that found in parallel cultures supplemented with acetate in concentrations of 11.6, 46.3 and 89.2 mM per 1000 ml. respectively. From the results presented in Figure 1 it will be seen that acetate has a marked inhibiting effect on the oxido-reduction process carried out by growing and multiplying cells of *D. desulphuricans*. The action of acetate manifests itself in prolonging the period of cellular adjustment, in retarding the rate of oxido-reduction in the phase of rapid cell multiplication, and in decreasing the maximum amounts of lactate and sulphate consumed as well as those of acetate and sulphide produced within reasonable experimental times.

In this experiment pH varied within the limits of 7.0 and 7.5 during the course of fermentation.

Experiment 2

This experiment was designed to show the influence of propionate on the sulphate reduction by *D. desulphuricans* in presence of lactate as a hydrogen donator. The concentrations of propionate tested were 7.4, 46.8 and 97.3 mM per 1000 ml. of substrate. From the results recorded in Figure 2 it may be concluded that propionate exhibits a combination of inhibitory effects similar to that observed after addition of acetate, thus lengthening the phase of cellular adjustment, slowing down the maximum rate of oxido-reduction and, in higher concentrations, reducing the final degree of metabolism.

The propionate added was not attacked at all, the amounts found on analyzing the culture substrates at different time intervals being on an average 7.0 ($\delta=0.4$, $n=9$), 46.9 ($\delta=0.3$, $n=9$) and 97.5 ($\delta=0.2$, $n=10$) mM per 1000 ml.

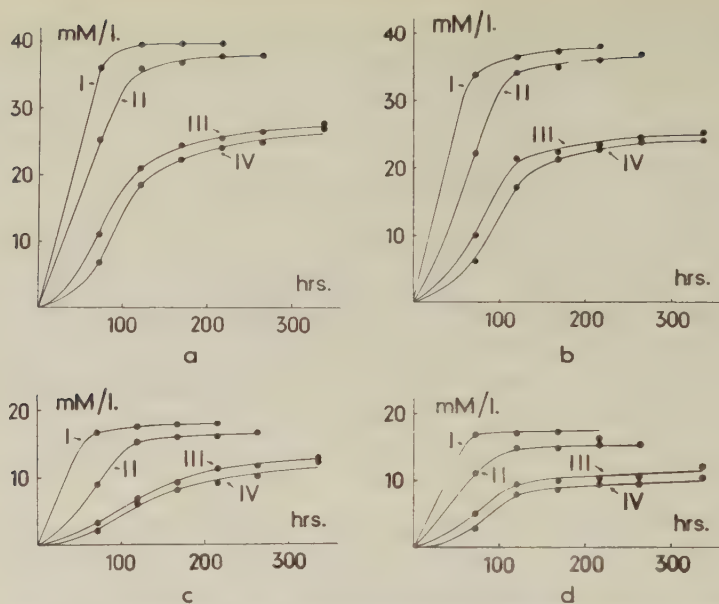


Figure 1. Effect of addition of acetate on the rate of sulphate reduction in lactate cultures of the Zürich strain of *D. desulphuricans*.

Initial concentration of lactate = 40.9 mM/l. Initial concentration of sulphate = 34.6 mM/l.

a. Lactate consumed.

b. Acetate produced.

c. Sulphate consumed.

d. Sulphide produced.

I. No addition of acetate.

II. Acetate added = 11.6 mM/l.

III. Acetate added = 46.3 mM/l.

IV. Acetate added = 89.2 mM/l.

During the course of fermentation the pH of the culture media varied between 6.6 and 8.1.

Experiment 3

In this experiment we intended to study the effect of n-butyrate on the metabolism of *D. desulphuricans* in the lactate-sulphate medium. Further it was interesting to compare this effect to that exerted by acetate and propionate under identical experimental conditions. Moreover, the experiment was designed to give some preliminary information as regards the influence of the nature of the inoculum upon the course of sulphate reduction in presence of short chain fatty acids. The concentrations of n-butyrate tested were 10.6,

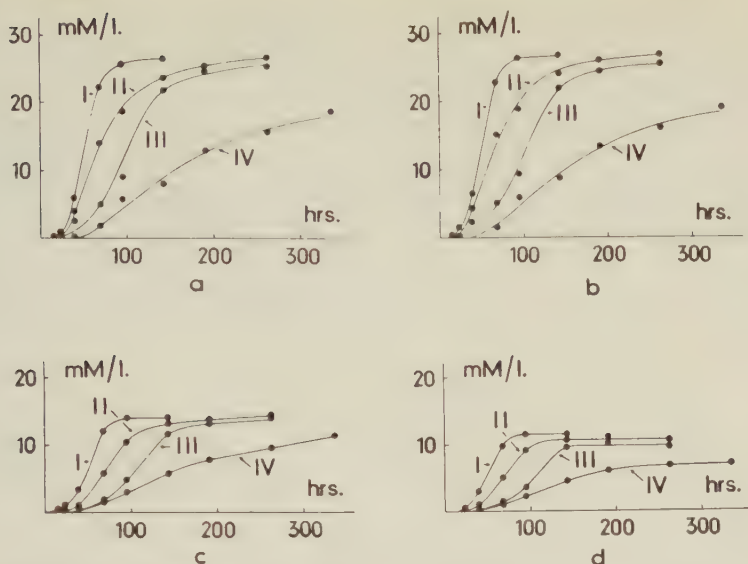


Figure 2. Effect of addition of propionate on the rate of sulphate reduction in lactate cultures of the Zürich strain of *D. desulphuricans*.

Initial concentration of lactate = 27.0 mM/l. Initial concentration of sulphate = 27.8 mM/l.

a. Lactate consumed.

b. Acetate produced.

c. Sulphate consumed.

d. Sulphide produced.

I. No addition of propionate.

II. Propionate added = 7.4 mM/l.

III. Propionate added = 46.8 mM/l.

IV. Propionate added = 97.3 mM/l.

50.3 and 100.3 mM per 1000 ml. of substrate. Acetate and propionate were added in amounts of 44.5 and 45.4 mM respectively per 1000 ml. In seeding the media a subculture grown for 20 hrs. was substituted for the 48 hrs. old cultures used in Experiments 1 and 2.

As shown in Figure 3 a very strong inhibition of oxido-reduction takes place in the cultures supplemented with *n*-butyrate, the phase of cellular adjustment (initial stationary phase + phase of accelerating growth and multiplication rate) being lengthened over a long period of time and the maximum rate of lactate oxidation and sulphate reduction being retarded considerably. As a matter of fact the test-organism practically failed to make any growth in the media supplied with 50.3 and 100.3 mM of *n*-butyrate respectively per 1000 ml.

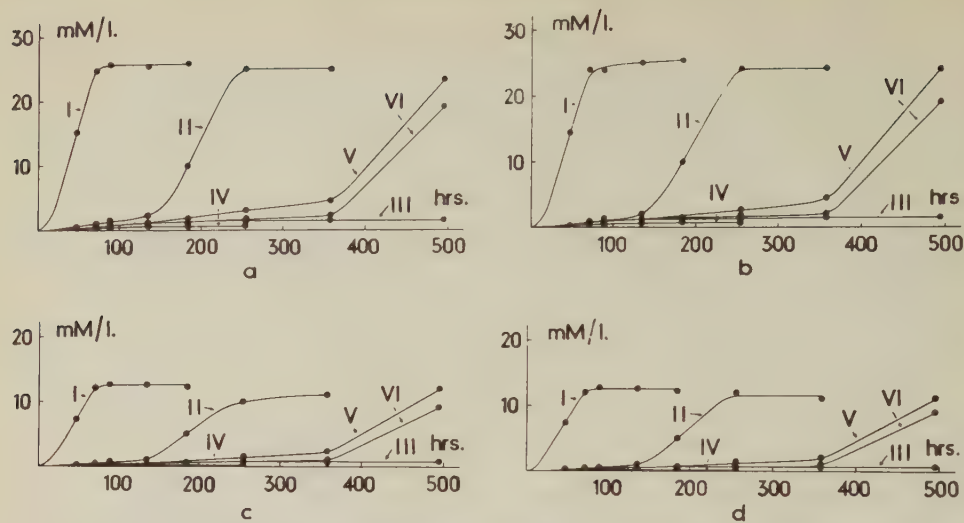


Figure 3. Effect of addition of *n*-butyrate, acetate and propionate on the rate of sulphate reduction in lactate cultures of the Zürich strain of *D. desulphuricans*. Initial concentration of lactate=32.5 mM/l. Initial concentration of sulphate=34.3 mM/l.

- a. Lactate consumed.
- b. Acetate produced.
- c. Sulphate consumed.
- d. Sulphide produced.

- I. No addition of fatty acid.
- II. *n*-Butyrate added= 10.6 mM/l.
- III. *n*-Butyrate added= 50.3 mM/l.
- IV. *n*-Butyrate added=100.3 mM/l.
- V. Acetate added=44.5 mM/l.
- VI. Propionate added=45.4 mM/l.

The analyses made at different intervals of time showed conclusively that the bacterial strain tested is incapable of utilizing *n*-butyric acid as a hydrogen donator or source of carbon, the concentration of this acid remaining constant over the whole period of fermentation.

Comparing the inhibitions produced on the metabolism of *D. desulphuricans* in the cultures with addition of fatty acids in approximately equal concentrations, it appears that *n*-butyrate is more toxic than acetate and propionate (50.3, 44.5 and 45.4 mM respectively per 1000 ml. of medium; curves III, V and VI).

As far as the interference of the inoculum with the short chain fatty acids and time is concerned a comparison of the results of Experiment 3 with those

of Experiments 1 and 2 indicates that the prolongation of the period of cellular adjustment induced by the acids at the same concentrations are much more pronounced after inoculation with material from a young culture (grown for 20 hrs.) containing a comparatively small number of cells than after seeding with material from an older culture (48 hrs.). As regards the extent of the retardation of maximum rate of oxido-reduction on the other hand no influence due to the different age and growth stage of the subcultures used for inoculation was observed.

The extreme pH values found in this experiment during the course of fermentation were 6.0 and 7.6.

Discussion

It is beyond the scope of this work to deal with a detailed analysis of the nature of the mechanism for the inhibition exerted by the short chain fatty acids on the sulphate reduction by *D. desulphuricans* in presence of lactate as a hydrogen donator. An attempt will be made, however, to discuss this mechanism taking into consideration our knowledge about the chemical and physical properties of the fatty acids, some general views on the nature of the physiological, particularly antimicrobial action of these compounds and recent theories on the nature of cellular uptake of molecules and ions.

The lower fatty acids are weak acids having an average pK value of about 4.8. This means that they exist to a great extent as undissociated molecules (free acid) at low pH values but as anions (highly dissociated salt) at high pH values. The undissociated molecules are more or less lipid soluble and pass into the cells through the lipid layer of the cytoplasmic membrane at a rate dependent on their solubility therein and on their outside concentration. Therefore the power of the fatty acids to enter cells by the lipid route is comparatively strong in acid solutions but weak in neutral or alkaline solutions (cf. Höber 1945). As mentioned above the initial pH of the culture media used in Experiments 1—3 varied between 7.0 and 7.5. Hence, it seems probable that the fatty acids tested only to a small extent penetrate into the cells of *D. desulphuricans* along the path formed by the lipid components of the surface membrane.

It is well known that a great number of organic substances are able to enter living cells, though their molecules are practically lipid insoluble. Further, it is a well-established fact that mineral salts are absorbed from the medium as ions, not as ion pairs or undissociated molecules (cf. Lundegårdh 1935, Höber 1945). In order to explain these phenomena which conclusively show the inadequacy of the Meyer-Overton lipid theory, another theory, viz. the pore or sieve theory, has been proposed. It states that the plasma membrane resembles a sieve or an ultrafilter with

pores of various widths allowing the lipid insoluble substances to pass into the cell according to the diameter of their molecules. On the suggestion of Collander and Bärklund the two theories have been combined into one, the lipid-sieve theory. It presents the plasma membrane as a mosaic composed of lipid areas and porous or sieve-like areas. The extent of lipid and porous surface on a membrane in addition to the chemical and physical properties of the lipid substance and the assortment of graded pore sizes control the nature of cell permeability, either the lipid solubility or the molecule diameter being the dominant factor in considering the way of entrance of a substance into the cell (cf. Höber 1945). Paying regard to the comparatively low lipid solubility of the lower fatty acids with a chain not longer than C₄ and further to the fact that the penetration of these compounds into the cell along the lipid path is difficult at the high pH values of the media used in the present investigation, we may assume that the acids tested enter the cells of *D. desulphuricans* mostly by passing as anions across the pores of the plasma membrane. Now it must be noted, however, that bacteria normally carry a negative charge under physiological conditions of pH. Further, detailed studies of the electrokinetic properties of bacteria have shown that young cells are more electronegative than adult cells (cf. Dubos 1947, Knaysi 1951 b). Not only the surface of the bacterial cells is negatively charged but their protoplasm as a whole due to its high content of nucleoproteins (50—80 per cent of the dry matter; cf. Knaysi 1951 a) and the very great proportion of free or bound ribonucleic acid in the cytoplasm, the concentration of this acid being at its maximum in growing and multiplying cells under favourable nutrient conditions (cf. Knaysi 1951 b). Owing to the negative charge of the surface and cytoplasm of bacteria cations may be taken up from the medium and accumulated in the cells by adsorption and ion exchange. In fact cationic exchange reactions between living cells and their liquid medium have been conclusively demonstrated in experiments with various bacteria, algae, protozoa, and roots of higher plants. On addition of inorganic salts to the medium the cells are capable of adsorbing the metallic cations in exchange for the hydrogen ions present in the surface layer of the cell membrane. The cations thus adsorbed may be replaced by a number of other positively charged ions on supplementing the medium with the respective salts, the extent of ionic replacement being controlled by the concentration of the ions and by their degree of adsorbability (cf. Dubos 1947, Lundegårdh 1935, 1939, 1940 a, 1945, 1947). Of course the mechanisms mentioned do not apply only to cations but also to anions. Thus the anions of mineral salts may be adsorbed by bacteria in exchange of hydroxyl or bicarbonate ions present in the surface layer of the cells. Owing to the dominating negative charge of the surface membrane and cytoplasm of bacterial cells the uptake and accumulation of anions cannot, however, take place as «passively» as outlined above, but require supply of energy liberated in metabolic reactions. On basis of results obtained in experiments on the mineral nutrition of wheat roots Lundegårdh and Burström have proposed the theory of anion respiration (Lundegårdh and Burström 1933, Lundegårdh 1935, 1937, 1939, 1940 a and b, 1945, 1947). According to this theory the metabolic energy used up in the processes of «active» uptake and accumulation of anions by root cells against the potential and concentration gradients is furnished by aerobic respiration of sugar. The anion respiration is supposed to be a complete oxidation of the sugar yielding carbon dioxide and water. The energetic coupling of this process with the uptake and accumulation of anions is proved by the sensitivity of both reactions to the inhibiting action of cyanide and azide (Lundegårdh 1945, 1950). The experimental data

indicate the existence of a low, active anion absorption in wheat roots even under anaerobic conditions. According to Lundegårdh (1940 a) it seems probable, however, that the energy liberated in anaerobic dissimilation of sugar is too small to serve for running an anion uptake and accumulation of practical importance to the wheat seedlings. Finally it may be noticed that the idea of active transfer, accumulation and retention of salts in plant and animal tissues against the concentration gradient, utilizing the fraction of the metabolic energy which is released in cyanide-susceptible aerobic (O_2 -consuming) dissimilation of carbohydrates, has been proposed also by Steward, Hoagland, Broyer and other authors (cf. Höber 1945, Lundegårdh 1947). In recent investigations Lundegårdh (1951 a and b, 1952) has demonstrated the presence of the cytochrome-cytochrome oxidase system in living roots of wheat and corn and its relation to the anion respiration and the active absorption of salts by the roots under aerobic conditions.

As mentioned above the anaerobic breakdown of sugar (fermentation) is considered an insufficient source of energy for a normal functioning of the mechanism of active transfer, accumulation and retention of ions in cells of wheat roots. In this connection it must be kept in mind, however, that the roots examined are parts of strongly aerobic organisms. In our view the very low efficiency of the active absorption of anions by wheat roots under anaerobic conditions must not necessarily be interpreted in terms of energy as a specific effect produced directly upon the process of ion uptake, but may also be due to a nonspecific effect manifesting itself as an injury to the cytoplasm or structures of the aerobic cells in general. Moreover, the aerobic root systems may be deficient in substances capable of acting as *chemical mediators* (van Niel, Lipmann, Kalekar; cf. Umbreit 1951) in the energetic coupling of fermentation (anaerobic dissimilation of sugar) and active anion absorption. In other words, the aerobic root cells contain the specific compounds capable of absorbing the energy released in aerobic respiration of sugar, storing this energy in their own structures (with formation of energy-rich bonds), and furnishing it to the physiological work done in active transfer, accumulation and retention of anions. The aerobic cells do not contain, however, the corresponding compounds linking the last processes to fermentation.

Applying the modern concepts of cell permeability and physiological energetics as reviewed above to the particular problem discussed in the present work, we may assume that the fatty acids tested enter the negatively charged cells of *D. desulphuricans* mostly by active absorption of their anions from the approximately neutral medium. This process is supposed to utilize some fraction of the energy released in fermentation, the strictly anaerobic cells of *D. desulphuricans* containing the chemical mediators essential for the energetic coupling of anaerobic dissimilation with active anion uptake. Assuming the lipid-sieve theory of structure of plasma membrane to be valid, the active anion absorption takes place in the porous areas of the cell surface (cf. above).

In our discussion we have so far assumed that the inhibitory effect produced by short chain fatty acids upon growth of the Zürich strain of *D. desulphuricans* is due to a toxic action by molecules and ions of these acids be-

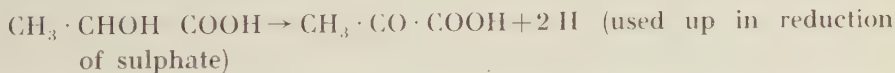
ing present inside the cytoplasmic membrane. This assumption seems reasonable in view of the following facts: The lower fatty acids with a carbon chain not longer than C_5 are anionic surface active agents causing lytic effects upon erythrocytes not stronger than those obtained with ammonium chloride (cf. Höber 1945). Lowering of pH favours the lytic influence, whereas an increase in pH weakens it. This corresponds to the lipid solubility of the undissociated molecules and to the increase in their concentration with decreasing pH. Further, it has been shown conclusively in experiments with various molds (*Aspergillus*, *Penicillium*, *Rhizopus*), yeasts (*Saccharomyces*, *Torula*) and bacteria (*Lactobacillus*, *Proteus*, *Sarcina*, *Staphylococcus*) that the fungistatic and bacteriostatic action of acetate, propionate and n-butyrate varies strongly with pH of the culture substrate, being greater at low than at higher pH values. Concentrations of the short chain fatty acids stopping growth of a certain organism completely at pH values of 3.0 to 4.0 do not induce any retardation at all in rate of growth of this organism at a pH of 6.0 to 7.0 (Hoffman, Schweitzer and Dalby 1939, Wolford and Andersen 1945; cf. also Heseltine 1952). Thus there are microorganisms in which the growth-inhibiting action of the lower fatty acids varies in proportion to the lipid solubility and lytic influence as related to the effect of pH of the media. As just mentioned these organisms are, however, not or only slightly susceptible to acetic, propionic and n-butyric acids within the pH range 7.0—7.5 while the Zürich strain of *D. desulphuricans* shows a very high sensitivity in this pH range in so far as lengthening of the period of cellular adjustment and decrease in rate of sulphate reduction in the phase of rapid cell multiplication is concerned. This speaks in favour of the view that the toxic effect exerted by the lower fatty acids upon the organisms examined by Hoffman et al. (1939) and Wolford and Andersen (1945) differs from that produced on the cells of *D. desulphuricans* with respect to the mechanism for penetration of the cytoplasmic membrane. However, in both cases it seems probable that the inhibiting action may be ascribed to molecules or anions of the acids being present in the interior of the cells. This assumption is further supported by the fact that a closely related organism (*D. rubent-schickii*; cf. introduction) is capable to utilize the same acids as hydrogen donors in reduction of sulphate to sulphide in neutral or slightly alkaline media. Moreover, the strictly anaerobic methane bacteria are able to break down the short chain fatty acids at approximately neutral pH values by decarboxylation (acetic acid) and by an oxido-reduction process analogous to that of the sulphate reduction, viz. the transformation of carbon dioxide to methane, the fatty acids (acetic, propionic and n-butyric acids) acting as donors and carbon dioxide (bicarbonate ion) as acceptor of hydrogen (Barker 1936 a and b, 1949). The formation of sulphide and methane from sul-

phate and carbon dioxide respectively are undoubtedly intracellular processes (cf. Barker 1949). The concerned organisms are probably capable of active absorption of anions from neutral and slightly alkaline solutions (pH 7.0—8.6; cf. Postgate 1951).

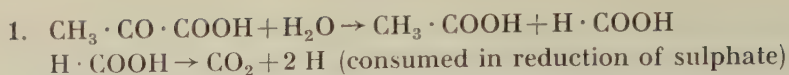
In connection with our discussion of the mechanism of the inhibiting action of the short chain fatty acids on growth of the Zürich strain of *D. desulphuricans* at neutral and alkaline pH values, we cannot neglect considering the conception of specific long-range enzyme forces postulated by Rothen (1947, 1948). According to this author enzymes and substrate proteins might react specifically, although separated by screens of inert material having a thickness of 200 Ångström units. In so far as the physiological activity of bacteria is concerned this would mean that enzymes present in the interior of the cells might be able to act through the thin cell membrane on substrate molecules in the surrounding medium. Besides the fact that the substrate molecules involved in the process of bacterial sulphate reduction are micromolecules, the hypothesis of interaction between enzymes and their substrates through screening films by means of specific long-range forces seems to be doubtful at this time in view of the critical evidence presented by several authors showing that a close contact of the enzyme with the substrate is effected under the experimental conditions applied (Karush and Siegel 1948, Iball 1949, Trurnit 1950, Singer 1950).

In trying to localize the site of the growth-inhibiting action exerted by the lower fatty acids after their passage into the cells of *D. desulphuricans*, we are faced with great difficulties because of the fact that the protoplasm contains an immense number of highly reactive components, rendering the interference by a great many mechanisms possible and also minimizing the chances of easily finding the cell functions most susceptible to the inhibiting action of the agents tested. Further, at present very little is known about the specific enzyme systems involved in the process of bacterial sulphate reduction with lactate as a hydrogen donator. Therefore we must restrict our discussion to speculations on some likely mechanisms for bacteriostatic and bactericidal action.

Senez (1951) has demonstrated that pyruvic acid is utilized by *D. desulphuricans* as a source of carbon for growth and as a donator of hydrogen for sulphate reduction. This indicates that pyruvate may act as an intermediate in the lactate metabolism:



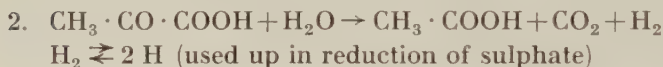
The pyruvate thus formed may be converted to acetic acid via various reactions, e.g.:



The anaerobic splitting of pyruvate to acetate and formate was originally supposed to be a hydroclastic reaction as expressed above. Later it was considered to be a phosphoroclastic reaction involving the formation of acetyl phosphate as an intermediate (Utter and Werkman 1944, Utter, Lipmann and Werkman 1945):



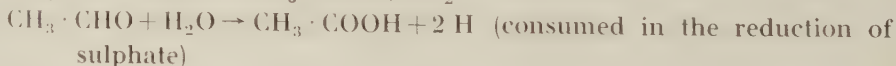
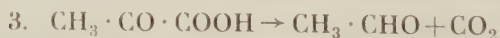
It has now been shown to require the presence of coenzyme A in its sulphhydryl form (HSCoA) and a phosphotransacetylase (Lipmann, Kaplan, Novelli, Tuttle and Guirard 1947, 1950, Kaplan and Lipmann 1948, Chantrenne and Lipmann 1950, Stadtman and Barker 1950, Korkes, del Campillo, Gunsalus and Ochoa 1951, Stadtman, Novelli and Lipmann 1951, Lynen and Reichert 1951, Lynen, Reichert and Rueff 1951, Stadtman 1952, Lipmann 1953):



The process of anaerobic cleavage of pyruvate to acetate, carbon dioxide and hydrogen has been postulated because of the fact that certain bacteria, e.g. *Clostridium butylicum*, break down pyruvate with formation of these end-products, although they are incapable of liberating hydrogen from formate (Koepsell and Johnson 1942, Koepsell, Johnson and Meek 1944, Wilson, Krampitz and Werkman 1948). In this type of anaerobic pyruvate decomposition formate apparently does not act as an intermediate. The reaction requires phosphate and is considered to be a phosphorolysis of pyruvate yielding acetyl phosphate and gas:

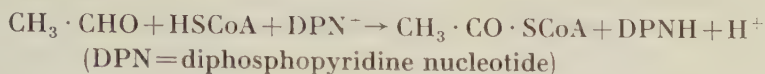


In view of the close relationship of coenzyme A to the «active acetate», «active 2-carbon residue» or «labile phosphate» it seems probable that this enzyme plays a rôle in the mechanism of phosphoroclastic breakdown of pyruvate resulting in acetate, carbon dioxide and hydrogen.



Assuming that acetaldehyde arising in decarboxylation of pyruvate (diphosphothiamine as a coenzyme) acts as a donator of hydrogen, coenzyme A

might be supposed to be involved in the reduction of sulphate. It has been shown that the aldehyde dehydrogenase present in cell extracts of the strict anaerobe *Clostridium kluverii* catalyzes the following reaction (Burton and Stadtman 1953, Lipmann 1953, Stadtman and Stadtman 1953, Anfinsen and Kielley 1954):



The diphosphopyridine nucleotide system might then act as a hydrogen donator through a chain of reactions linking it to the process of sulphate reduction:

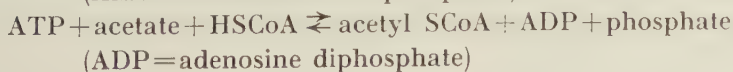
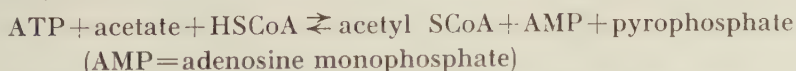


Several authors working with extracts of cells of animal tissues and facultative anaerobes (*Escherichia coli*, *Streptococcus faecalis*) have agreed that the over-all reaction of α -keto acid oxidation may be formulated as shown above (Korkes, del Campillo, Gunsalus and Ochoa 1951, Korkes, del Campillo and Ochoa 1952, Ochoa and Stern 1952, Littlefield and Sanadi 1952, Lipmann 1953, Slator 1953, Anfinsen and Kielley 1954). In presence of phosphotransacetylase and orthophosphate acetyl phosphate is formed:



Diphosphothiamine is required in addition to coenzyme A, diphosphopyridine nucleotide and an acetyl acceptor system. This suggests that decarboxylation of pyruvate to acetaldehyde (reaction 3) might be an intermediate reaction (Ochoa and Stern 1952, Slator 1953).

From the findings summarized above it is evident that coenzyme A in its sulfhydryl form plays an important rôle in the anaerobic conversion of pyruvate to acetate and carbon dioxide, acetyl coenzyme A functioning as an intermediate in this reaction. In the cells of microorganisms the acetylation of this coenzyme may, however, also be brought about in presence of acetate and adenosine triphosphate (ATP) as follows (Lipmann, Jones, Black and Flynn 1952, Lipmann 1953, Stadtman and Stadtman 1953, Anfinsen and Kielley 1954):



Moreover, the butyryl derivative of coenzyme A may be synthesized in presence of butyrate and CoA transphorase catalyzing the following reaction (Stadtman 1953, Stadtman and Stadtman 1953, Lipmann 1953, Anfinsen and Kielley 1954):



It has been shown that the propionyl derivative may be formed by way of an analogous reaction:



In view of the fact that the above reactions take place in cell extracts of strictly anaerobic bacteria, the possibility of a competition between the fatty acids and pyruvate for the sulphhydryl group of coenzyme A might be taken into consideration as a working hypothesis in investigations on the growth-inhibiting effect exerted by acetate, propionate and n-butyrate on the cells of *D. desulphuricans*. It is well known that in cases of competitive inhibition the degree of the effect is related not only to the concentrations of substrate (lactate and pyruvate) and inhibitor (acetate, propionate and n-butyrate) but also to the total amount of enzyme present in the cells incorporated into the media as inoculum. Further, the inhibitory effect is dependent on the affinity of the substrate and that of the inhibitor for the enzyme molecules. This in addition to the particular difficulties met with in considering the different factors controlling cell permeability makes a great number of carefully planned experiments necessary for solving the problems outlined in the present discussion.

Summary

The present work deals with the effects produced by short chain fatty acids on sulphate reduction in proliferating cultures of a freshly isolated strain of *Desulphovibrio desulphuricans*.

All experiments were carried out with strictly anaerobic cultures (oxygen-free nitrogen, addition of Mohr's salt, mercury seal tube) grown at a temperature of 37° C on the synthetic lactate-sulphate medium of Starkey (1938), modified as to contain 27—41 mM of lactic acid and 27—35 mM of sulphate per 1000 ml. The initial pH was adjusted to 7.0—7.5. The extreme pH values observed during the course of fermentation were 6.0 and 8.1. The fatty acid concentrations tested amounted to 11.6—89.2 mM of acetic acid, 7.4—97.3 mM of propionic acid and 10.6—100.3 mM of n-butyric acid per 1000 ml. of culture substrate.

The rate of metabolism was estimated by analyzing the culture substrates at intervals for sulphate, sulphide, lactic acid and fatty acids (acetic acid, acetic acid + propionic acid, acetic acid + n-butyric acid).

Under the experimental conditions applied the Zürich strain of *D. desulphuricans* is incapable of utilizing the fatty acids tested as a source of carbon for growth or as a donator of hydrogen for sulphate reduction.

Acetate, propionate and n-butyrate have a marked inhibiting action on the sulphate reduction carried out by growing and multiplying cells of *D. desulphuricans* in presence of lactate as a carbon source and hydrogen donator. The inhibitory effect manifests itself in prolonging the period of cellular adjustment (initial stationary phase + phase of accelerating growth and multiplication rate), in retarding the rate of oxido-reduction in the phase of rapid cell multiplication (maximum rate) and in some cases in decreasing the maximum amounts of lactate and sulphate consumed as well as those of acetate and sulphide produced within reasonable experimental periods. In cultures supplied with single fatty acids in approximately equal concentrations the inhibition produced with n-butyrate is stronger than that observed in presence of acetate or propionate.

The inhibitory effects thus observed were discussed in the light of our knowledge about the chemical and physical properties of the short chain fatty acids (lipoid solubility, dependence on electrolytic dissociation) and recent theories on cell permeability and its relation to the chemical and physical structure of the cell membrane (lipoid theory of Meyer and Overton, lipoid-pore or lipoid-sieve theory of Collander and Barlund, the theory of anion respiration and active anion uptake proposed by Lundegårdh and Burström). Further, some general views on the nature of enzyme and inhibitor action (action of chemical mediators in energetic coupling of enzymatic reactions, intracellular action with intimate contact between enzyme and substrate, existence of specific long range enzyme forces according to Rothen, anionic surface active agents, theory of competitive inhibition) and the modern theories on the mechanism of lactate and pyruvate dissimilation under anaerobic conditions (action of coenzyme A in its sulphydryl form according to Lipmann, Lynen, Barker, Stadtman, Gunsalus, Ochoa and others) were taken into consideration. On basis of the discussion a working hypothesis was proposed about the mechanism of the inhibiting action exerted by the lower fatty acids on growth and sulphate reduction in cultures of *D. desulphuricans* on lactate media at approximately neutral pH values. According to this hypothesis the short chain fatty acids are adsorbed on the cell surface by anion exchange. They enter the cytoplasm of the negatively charged cells as anions across the porous areas of the cell membrane. The energy used up in this active uptake of anions against a potential gradient is furnished by ferment-

tation, factors capable of acting as chemical mediators in the energetic coupling of the two processes under strictly anaerobic conditions being present in the cells. It is further assumed that the intracellular conversion of lactate to acetate proceeds via pyruvate as an intermediate. The formation of acetate from pyruvate is supposed to be catalyzed by coenzyme A in its sulfhydryl form, the acetyl derivative of the coenzyme functioning as a precursor of acetate. In presence of adenosine triphosphate this mercaptoester may, however, also be synthesized from acetate. Further the corresponding propionyl and butyryl derivatives of coenzyme A may be formed from propionate and butyrate, respectively, by the action of a transphorase system. This suggests that the inhibition of sulphate reduction observed in cultures of *D. desulphuricans* supplemented with acetate, propionate and n-butyrate might be due to a competition between the fatty acids and pyruvate for the sulfhydryl group of coenzyme A.

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References

- Anfinsen, C. B. & Kielley, W. W.: Biological oxidations. — *Ann. Rev. Biochem.* 23: 17. 1954.
Baars, J. K.: Over sulfaatreductie door bacteriën. — *Diss.*, Delft 1930.
Barker, H. A.: On the biochemistry of the methane fermentation. — *Arch. f. Mikrobiol.* 7: 404. 1936 a.
— Studies upon the methane-producing bacteria. — *Arch. f. Mikrobiol.* 7: 420. 1936 b.
— Le métabolisme des gaz par les bactéries anaérobies. — *Ann. Inst. Past.* 77: 361. 1949.
Burton, R. M. & Stadtman, E. R.: The oxidation of acetaldehyde to acetyl coenzyme A. — *Journ. Biol. Chem.* 202: 873. 1953.
Chantrenne, H. & Lipmann, F.: Coenzyme A dependence and acetyl donor function of the pyruvate-formate exchange system. — *Journ. Biol. Chem.* 187: 757. 1950.
Dubos, R. J.: The bacterial cell. — Cambridge, Mass. 1947.
Eichholtz, F.: Sauerkraut und ähnliche Gärerzeugnisse. — *Die Wissenschaft. Band 96*, Braunschweig 1941.
Elsden, S. R.: The application of the silica gel partition chromatogram to the estimation of volatile fatty acids. — *Biochem. Journ.* 40: 252. 1946.
Friedemann, T. E.: The identification and quantitative determination of volatile alcohols and acids. — *Journ. Biol. Chem.* 123: 161. 1938.
— Colónio, M. & Shaffer, P. A.: The determination of lactic acid. — *Journ. Biol. Chem.* 73: 335. 1927.

- Gregory, J. D., Novelli, G. D. & Lipmann, F.: The composition of coenzyme A. — *Journ. Amer. Chem. Soc.* 74: 854. 1952.
- Heseltine, W. W.: Sodium propionate and its derivatives as bacteriostatics and fungistatics. — *Journ. Pharm. Pharmacol.* 4: 577. 1952.
- Hill, C. H.: Studies on the inhibition of growth of *Streptococcus faecalis* by sodium propionate. — *Journ. Biol. Chem.* 199: 329. 1952.
- Höber, R.: Physical chemistry of cells and tissues. Philadelphia and Toronto 1945.
- Hoffman, C., Schweitzer, T. R. & Dalby, G.: Fungistatic properties of the fatty acids and possible biochemical significance. — *Food Research* 4: 539. 1939.
- Hoppe-Seyler, F.: Ueber die Gährung der Cellulose mit Bildung von Methan und Kohlensäure. — *Zeitschr. f. physiol. Chemie* 10: 401. 1886.
- Iball, J.: Antigen films and long-range forces. — *Science* 109: 18. 1949.
- Kaplan, N. O. & Lipmann, F.: The acetyl precursor in pyruvate synthesis in *Escherichia coli*. — *Journ. Biol. Chem.* 176: 459. 1948.
- Karush, F. & Siegel, B. M.: The structure of antigen films and long-range forces. — *Science* 108: 107. 1948.
- King, T. E. & Cheldelin, V. H.: Pantothenic acid studies. IV. Propionic acid and β -alanine utilization. — *Journ. Biol. Chem.* 174: 273. 1948.
- Knaysi, G.: Chemistry of the bacterial cell. *Bacterial Physiology* (edited by Werkman, C. H. & Wilson, P. W.): 1. New York 1951 a.
- The structure of the bacterial cell. — *Bacterial Physiology* (edited by Werkman, C. H. & Wilson, P. W.): 28. New York 1951 b.
- Koepsell, H. J. & Johnson, M. J.: Dissimilation of pyruvic acid by cell-free preparations of *Clostridium butylicum*. — *Journ. Biol. Chem.* 145: 379. 1942.
- & Meek, J. S.: Rôle of phosphate in pyruvic acid dissimilation by cell-free extracts of *Clostridium butylicum*. — *Journ. Biol. Chem.* 154: 535. 1944.
- Kolthoff, I. M. & Sandell, E. B.: Textbook of quantitative inorganic analysis. — New York 1947.
- Korkes, S., del Campillo, A., Gunsalus, I. C. & Ochoa, S.: Enzymatic synthesis of citric acid. IV. Pyruvate as acetyl donor. — *Journ. Biol. Chem.* 193: 721. 1951.
- & Ochoa, S.: Pyruvate oxidation system of heart muscle. — *Journ. Biol. Chem.* 195: 541. 1952.
- Lipmann, F.: On chemistry and function of coenzyme A. — *Bact. Rev.* 17: 1. 1953.
- Kaplan, N. O., Novelli, G. D., Tuttle, L. C. & Guirard, B. M.: Coenzyme for acetylation, a pantothenic acid derivative. — *Journ. Biol. Chem.* 167: 869. 1947.
- — — — Isolation of coenzyme A. — *Journ. Biol. Chem.* 186: 235. 1950.
- Jones, M. E., Black, S. & Flynn, R. M.: Enzymatic pyrophosphorylation of coenzyme A by adenosine triphosphate. — *Journ. Amer. Chem. Soc.* 74: 2384. 1952.
- Littlefield, J. W. & Sanadi, D. R.: Rôle of coenzyme A and diphosphopyridine nucleotide in the oxidation of pyruvate. — *Journ. Biol. Chem.* 199: 65. 1952.
- Lundegårdh, H.: Theorie der Ionenaufnahme in lebende Zellen. — *Naturwiss.* 23: 313. 1935.
- Untersuchungen über die Anionenatmung. — *Biochem. Zeitschr.* 290: 104. 1937.
- An electro-chemical theory of salt absorption and respiration. — *Nature.* 143: 203. 1939.
- Investigations as to the absorption and accumulation of inorganic ions. — *Ann. Agric. Coll. Sweden* 8: 233. 1940 a.
- Anionenatmung und Blüten. — *Planta* 31: 184. 1940 b.
- Absorption, transport and exudation of inorganic ions by the roots. — *Ark. f. Bot.* (Stockholm) 32 A, N:o 12. 1945.

- Mineral nutrition of plants. — *Ann. Rev. Biochem.* 16: 503. 1947.
- Reversal of inhibition of respiration and salt absorption by cyanide and azide. — *Nature* 165: 513. 1950.
- Spectroscopic evidence of the participation of the cytochrome-cytochrome-oxidase system in the active transport of salts. — *Ark. f. Kemi (Stockholm)* 3: 69. 1951 a.
- The cytochrome-cytochrome oxidase system of living roots of wheat and corn. — *Ark. f. Kemi (Stockholm)* 3: 469. 1951 b.
- Properties of the cytochrome system of wheat roots. — *Arch. f. Kemi (Stockholm)* 5: 97. 1952.
- & Burström, H.: Untersuchungen über die Salzaufnahme der Pflanzen. III. — *Biochem. Zeitschr.* 261: 235. 1933.
- Lynen, F. & Reichert, E.: Zur chemischen Struktur der »aktivierten Essigsäure«. — *Angew. Chemie* 63: 47. 1951.
- — & Rueff, L.: Zum biologischen Abbau der Essigsäure. VI. »Aktivierte Essigsäure«, ihre Isolierung aus Hefe und ihre chemische Natur. — *Liebigs Ann. d. Chemie* 574: 1. 1951.
- Ochoa, S. & Stern, J. R.: Carbohydrate metabolism. — *Ann. Rev. Biochem.* 21: 547. 1952.
- Postgate, J. R.: The reduction of sulphur compounds by *Desulphovibrio desulphuricans*. — *Journ. gen. Microbiol.* 5: 725. 1951.
- Richard, O. & Heinzl, O.: Vergleichende mikrobiologische Untersuchungen an A.I.V.- und Amasil-Sauerfutter. — *Schweiz. Landw. Monatshefte* 20: 232, 269. 1942.
- Rothén, A.: Immunological reactions between films of antigen and antibody molecules. — *Journ. Biol. Chem.* 168: 75. 1947.
- Long range enzymatic action on films of antigen. — *Journ. Amer. Chem. Soc.* 70: 2732. 1948.
- Rubentschik, L.: Ueber Sulfatreduktion durch Bakterien bei Zellulosegärungsprodukten als Energiequelle. — *Zentralbl. f. Bakt. II. Abt.* 73: 483. 1928.
- Senez, J.: Etude comparative de la croissance de *Sporovibrio desulfuricans* sur pyruvate et sur lactate de soude. — *Ann. Inst. Past.* 80: 395. 1951.
- Singer, S. J.: On the unlikelihood of specific long range forces in immunologic and enzymatic reactions. — *Journ. Biol. Chem.* 182: 189. 1950.
- Slater, E. C.: Biological oxidations. — *Ann. Rev. Biochem.* 22: 17. 1953.
- Somm, H.: Ueber die Hemmung von Assimilation und Atmung durch niedere Fettsäuren und spezifische Enzymgifte bei *Mycelium radicis atrovisens* Melin. — *Schweiz. Zeitschr. f. allg. Path. Bakt.* 16: 851. 1953 a.
- Beiträge zur Kenntnis der Wachstums- und Atmungsphysiologie von *Mycelium radicis atrovisens* Melin mit besonderer Berücksichtigung der Stickstoffquelle und einiger Mono-, Di- und Tricarbonsäuren. — *Diss. ETH, Zürich* 1953 b.
- Stadtman, E. R.: The purification and properties of phosphotransacetylase. — *Journ. Biol. Chem.* 196: 527. 1952.
- The coenzyme A transphorase system in *Clostridium kluveri*. — *Journ. Biol. Chem.* 203: 501. 1953.
- & Barker, H. A.: Fatty acid synthesis by enzyme preparations of *Clostridium kluveri*. VI. Reactions of acyl phosphates. — *Journ. Biol. Chem.* 184: 769. 1950.
- Novelli, G. D. & Lipmann, F.: Coenzyme A function in and acetyl transfer by phosphotransacetylase system. — *Journ. Biol. Chem.* 191: 365. 1951.
- & Stadtman, T. C.: Metabolism of microorganisms. — *Ann. Rev. Microbiol.* 7: 143. 1953.
- Starkey, R. L.: A study of spore formation and other morphological characteristics of *Vibrio desulfuricans*. — *Arch. f. Mikrobiol.* 9: 268. 1938.

- Treadwell, F. P.: Kurzes Lehrbuch der analytischen Chemie. Band II. — Leipzig 1930.
- Trurnit, H. J.: Protein permeability through thin organic layers. — *Science* 111:1. 1950.
- Umbreit, W. W.: Significance of autotrophy for comparative physiology — *Bacterial Physiology* (edited by Werkman, C. H. & Wilson, P. W.): 566. New York 1951.
- Utter, M. F., Lipmann, F. & Werkman, C. H.: Reversibility of the phosphoroclastic split of pyruvate. — *Journ. Biol. Chem.* 158: 521. 1945.
- & Werkman, C. H.: Formation and reactions of acetyl phosphate in *Escherichia coli*. — *Arch. Biochem.* 5: 413. 1944.
- Wikén, T. & Ghose, T. K.: Stimulation of bacterial sulphate-reduction by (+)-biotin. — *Phys. Plant.* 7: 713. 1954.
- & Zobrist, F.: Ueber die Verwertung der Propionsäure durch Butylbakterien. — *Schweiz. Zeitschr. f. allg. Path. Bakt.* 16: 836. 1953.
- Wilson, J., Krampitz, L. O. & Werkman, C. H.: Reversibility of a phosphoroclastic reaction. — *Biochem. Journ.* 42: 598. 1948.
- Wolford, E. R. & Andersen, A. A.: Propionates control microbial growth in fruits, vegetables. — *Food Industries* 17: 622. 1945.

Effects of B-vitamins and Amino-acids on Nitrification

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Introduction

The nitrifying bacteria are able to synthesize their cell material entirely from inorganic compounds, i.e. they are true autotrophic organisms. However, as growth of the bacteria in liquid mineral medium and on silica-gels is rather scanty, it has been supposed that one or more growth factors might be essential. Now and then different workers (Winogradsky and Omeliansky, 1899; Murray, 1923; Hes, 1937) have reported that soil extract and other undefined organic infusions accelerate nitrification. Fred and Davenport (1921) found that a preparation of egg albumen ('Nährstoff-Heyden') stimulated nitrification when added to cultures of the bacteria, and later Kingma Boltjes (1934) observed that colonies of both *Nitrosomonas* and *Nitrobacter* on washed agar plates became larger when this compound was added. The effect of urine, yeast extract, soil extract, thiamine and β -indolylacetic acid of nitrification in mixed cultures of nitrifiers and heterotrophic bacteria was tested by Meiklejohn (1953), but no stimulation was observed. Lees (1954) noted an irregular reduction in the lag period of *Nitrosomonas* when biotin was added to the media.

The present investigation comprises the effect of a series of B-vitamins, amino-acids, potato extract, and sterilized and nonsterilized soil on nitrification by *Nitrosomonas europaea* in pure culture and in mixed culture with heterotrophic soil bacteria.

Materials and methods

Bacteria. A strain of *Nitrosomonas europaea* received from Dr. H. L. Jensen, The State Laboratory of Plant Culture, Lyngby, Denmark, was used for all pure culture experiments. The mixed cultures were the result of an attempt to isolate *Nitrosomonas* in pure culture from garden soil by ordinary dilution technique. By this procedure a stable population of ammonia-oxidizers of the *Nitrosomonas*-type and three heterotrophic bacteria was obtained. It was not possible to isolate *Nitrosomonas* from these cultures either by further dilution or by plating on mineral silica-gels. The heterotrophic bacteria could, however, easily be isolated from nutrient agar. The heterotrophs were studied in pure cultures and found to be (1) *Pseudomonas* sp. (ambiguous?), (2) *Pseudomonas* sp. (differing from (1) in being able to ferment maltose but not arabinose, and to acidify and coagulate litmus milk), and (3) *Hyphomicrobium vulgare*, previously found to live in close association with nitrifying bacteria (Stutzer and Hartleb, 1899; Kingma Boltjes, 1934).

Media. A basal mineral medium (A) was made up from $(\text{NH}_4)_2\text{SO}_4$: 4 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$: 0.125 g; NaCl : 0.125 g; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$: 0.0025 g; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$: 0.0025 g; Hoagland trace mineral solution 1 ml, tap water 1000 ml. A small amount of calcium carbonate was added to each culture vessel. The media were autoclaved at 150° for 20 min. Final pH was 8.0 (glass-electrode).

Another medium (B) was prepared from autoclaved medium A (minus CaCO_3) by addition of 1 per cent glucose (sterilized by filtration) and a small spatula of equal weights of calcium carbonate and garden soil previously sterilized by heating at 150° for 3 hours. This medium was used only for mixed culture experiments.

All cultures were grown in 20 mm wide test-tubes containing 4 ml of medium and incubated at 28° .

Vitamins. The following vitamins were tested: Thiamine (0.07 and 5 $\mu\text{g/ml}$), riboflavin (0.5 $\mu\text{g/ml}$), biotin (0.0003 $\mu\text{g/ml}$), p-aminobenzoic acid (2 $\mu\text{g/ml}$), nicotinamide (1 and 40 $\mu\text{g/ml}$), pyridoxin (1 $\mu\text{g/ml}$), pantothenic acid (0.0025 $\mu\text{g/ml}$), B_{12} (0.1 $\mu\text{g/ml}$), and a mixture of all eight vitamins together, the components being present in the same concentration as above (thiamine and nicotinamide in the lowest concentrations only). The vitamins were sterilized separately by autoclaving or by filtration through Seitz filters, and added aseptically to the media before inoculation.

Amino-acids. Glutamic acid, tryptophan, phenylalanine, tyrosine and histidine were tested. The amino-acids also were autoclaved separately and subsequently added to the media, giving a uniform concentration of 100 μg per ml.

Potato extract and soil. The potato extract was prepared from freshly harvested potatoes. 100 g of potato was minced with 100 ml of tap water in a Waring Blendor for 5 min. and the juice thus obtained was thereafter filtered, firstly through paper and finally through a Seitz filter giving a sterile, clear and light brown liquid. 0.2 ml of this extract was added to medium A.

Finally the effect of adding garden soil directly to the media was tested. Aliquots of 0.1 g of air-dried soil were partly autoclaved together with 4 ml of medium A, and partly added untreated to the sterile medium.

Nitrite estimations. The effect of the different compounds on nitrification was estimated by the rate of nitrite production by *Nitrosomonas*. Measurements were made 5 and 10 days after inoculation by reading the intensity of the Griess-Hosway reaction in a Coleman spectrophotometer. All figures in the tables represent averages of duplicate or triplicate cultures.

Table 1. *Effect of B-vitamins on nitrification.* — The vitamin components in the mixture was represented in the same concentration as when added alone. (Thiamine 0.07 µg/ml; nicotinamide 1 µg/ml).

Vitamin added	Conc. µg/ml	µg NO ₂ -N per ml formed in					
		pure cult. Med. A		mixed cult. Med. A		mixed cult. Med. B	
		5 days	10 days	5 days	10 days	5 days	10 days
None	—	20	80	20	95	20	65
Thiamine	0.07	20	85	20	95	15	30
id.	5	20	40	—	—	—	—
Riboflavin	0.5	20	85	15	90	15	65
p-aminobenzoic acid	2	25	80	15	90	15	65
Pyridoxin	1	20	85	15	80	20	40
Biotin	0.0003	15	70	25	90	15	45
Pantothenic acid	0.0025	20	100	15	80	20	50
B ₁₂	0.1	20	80	15	85	20	35
Nicotinamide	1	20	85	20	80	20	65
id.	40	25	60	—	—	—	—
B-vitamin mixture ...		15	60	20	90	20	60

Results

Table 1 shows that addition of B-vitamins to cultures of *Nitrosomonas* does not influence the rate of nitrification. Neither stimulation nor inhibition could be significantly detected in pure and mixed cultures in the inorganic medium (A). There seemed, however, to be some inhibition by 5 µg/ml thiamine, and a slight stimulation by pantothenic acid. In some cultures a significant stimulation was found when biotin and thiamine were added together, but the findings could not be reproduced. Further it should be mentioned that in recent experiments colony formation on washed agar media (Difco Special Agar Noble) seems to be accelerated when biotin is added.

It was supposed that a growth factor might be essential for one or more of the heterotrophs to produce another growth factor essential for the nitrifier, but different from the B-vitamins tested here. But as medium A will give only scanty growth of the heterotrophic bacteria this scheme could not be justified. The introduction of glucose as an organic carbon-source, as well as a small portion of soil, into the inorganic medium resulted in an abundant growth (with predominance of the maltose fermenting *Pseudomonas* sp.) in all cultures, but nitrification, as measured by nitrite production, was not stimulated. On the contrary nitrification seems to be depressed to some extent. Recent experiments with the inorganic nitrogen metabolism of the heterotrophic bacteria taking part in the mixed cultures show that all of them are able to metabolize nitrite when this ion is added to pure cultures in diluted broth. The apparent small depression of nitrite production in the

Table 2. *Effect of amino-acids on nitrification.*

Amino-acid added	Conc. µg/ml	µg NO ₂ -N per ml formed in			
		pure culture, med. A		mixed culture, med. A	
		5 days	10 days	5 days	10 days
None	—	30	100	30	105
Histidine	100	15	45	25	75
Tyrosine	100	0	0	0	55
Glutamic acid	100	0	30	5	30
Phenylalanine	100	0	0	10	55
Tryptophan	100	5	70	15	50

glucose-soil-media in the present experiments therefore could be due to removal of nitrite by the heterotrophs. No conclusions could therefore be drawn from the results obtained in medium B.

In the amino-acid experiment (Table 2) it is shown that all compounds tested inhibit nitrification in pure cultures of *Nitrosomonas*. At the concentrations employed, tyrosine and phenylalanine are so toxic to the organism that no nitrification takes place at all. The toxicity is, however, abolished in the mixed cultures, which could be explained as metabolism of these compounds by the heterotrophic bacteria. Glutamic acid and tryptophan added to pure cultures inhibited nitrification at first, as did tyrosine and glutamic acid in mixed cultures, but after 10 days the inhibiting effect seems to be reduced. Jensen (1950) found that 1.5 per cent (15.000 µg/ml) of glutamic acid added to cultures of *Nitrosomonas europaea* only slightly inhibited nitrification. However, the strain used in Jensen's experiments was isolated from manure and appeared to tolerate much higher concentrations of organic compounds than strains used by other workers. Lees (1952) found that histidine was highly toxic at a concentration of 5×10^{-3} M (775 µg/ml) in a 3 hours experiment with washed cell suspensions of *Nitrosomonas*. On the other hand,

Table 3. *Effect of potato extract and garden soil on nitrification in mixed cultures.*

Medium A.

Addition	µg NO ₂ -N per ml formed in	
	5 days	10 days
None	40	70
Potato extract	35	65
Soil (autoclaved)	35	85
id. (unsterilized)	50	75 ¹
id. (no other inoculum)	15	15 ¹

¹ Nitrate present

Lees found no inhibition with the same concentrations of tyrosine, glutamic acid, phenylalanine and tryptophan. As conditions in Lees experiments are different from those employed in the present investigation the results could not easily be compared. In the present experiment nitrification could take place and amount to 50 per cent in pure cultures with 100 μg histidine added per ml.

The addition of potato extract and soil to mixed cultures did not change the rate of nitrification. When the soil added was not sterilized, nitrate appeared in the cultures. This, of course, was expected as nitrite-oxidizing bacteria are normal habitants in garden soil.

Summary

Thiamine (0.07 and 5 $\mu\text{g}/\text{ml}$), riboflavin (0.5 $\mu\text{g}/\text{ml}$), biotin (0.0003 $\mu\text{g}/\text{ml}$), p-aminobenzoic acid (2 $\mu\text{g}/\text{ml}$), nicotinamide (1 and 40 $\mu\text{g}/\text{ml}$), pyridoxin (1 $\mu\text{g}/\text{ml}$), pantothenic acid (0.0025 $\mu\text{g}/\text{ml}$), B_{12} (0.1 $\mu\text{g}/\text{ml}$), and a mixture of these vitamins did not affect the nitrification of *Nitrosomonas* neither in pure culture nor in association with heterotrophic soil bacteria from enrichment cultures of the ammonia-oxidizing autotroph.

100 $\mu\text{g}/\text{ml}$ of tryptophan, glutamic acid and histidine slightly inhibited nitrification in pure cultures; tyrosine and phenylalanine were toxic and stopped nitrification entirely. When added to mixed cultures these amino-acids were less inhibitory, and after 10 days nitrite production amounted to 30—70 per cent of nitrite in the controls.

Potato extract and garden soil did not affect the rate of nitrification.

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References

1. Fred, E. B. & Davenport, A.: The effect of organic nitrogenous compounds on the nitrate-forming organism. — *Soil Sci.* 11: 389. 1921.
2. Hes, J. W.: Stoffwechselphysiologie von *Nitrosomonas*. — *Rec. Trav. Bot. Neerl.* 34: 234. 1937.
3. Jensen, H. L.: En stamme af *Nitrosomonas europaea* fra staldgodning. — *Tidsskr. f. Planteavl* 54: 62. 1950.
4. Kingma Boltjes, T. Y.: Onderzoekingen over nitrificerende bakterien. — Thesis, Delft. 1934.
5. Lees, H.: The biochemistry of the nitrifying organisms. I. The ammonia-oxidizing systems of *Nitrosomonas*. — *Biochem. J.* 52: 134. 1952.

6. Lees, H.: The biochemistry of the nitrifying bacteria. — *Autotrophic Microorganisms* (4th sympos. Soc. Gen. Microbiol.), Cambridge. P. 84. 1954.
7. Meiklejohn, Jane: Some organic substances and the nitrifying bacteria. — *Proc. Soc. Appl. Bact.* 15: 77. 1953.
8. Murray, T. J.: Food accessory factors and the nitrite bacteria. — *Proc. Soc. Exp. Biol.*, N.Y. 20: 301. 1923.
9. Stutzer, A. & Hartleb, R.: Untersuchungen über die bei der Bildung von Salpeter beobachteten Mikroorganismen. — *Zbl. Bakt.* 2. Abt. 5: 678. 1899.
10. Winogradsky, S. & Omeliansky, V.: Über den Einfluss der organischen Substanzen auf die Arbeit der nitrifizierenden Bakterien. — *Zbl. Bakt.* 2. Abt. 5: 329, 377, 429. 1899.

On the Cytochromes *b* and *dh* in the Roots of Cereals

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Introduction and experimental technique

The spectrophotometric investigation of intact roots of wheat and other cereals (corn, rye, rice) showed the existence of a complete cytochrome system, in which the components *a*, *c*, and *b* could be optically isolated. The analysis was aided by the fact that the steady states of the single cytochromes are not synchronously shifted if inhibitors are applied or if the conditions are changed from aerobical to anaerobical and *vice versa*. At an early date of these investigations the appearance of a band at 570–571 m μ after prolonged anaerobiosis indicated the presence of a new hemin component. During the continued analysis this component »PX 570« revealed itself as a true cytochrome, which apparently is not a member of the cytochrome *b* family, even if its prosthetic group is protohemin, too (Lundegårdh 1954 a). For this reason it was given the name cytochrome *dh*. This name indicated the fact that the new cytochrome has its place near the bottom of the »electron ladde« leading from the dehydrogenases to oxygen and it was in a preliminary communication suggested that the new cytochrome might be identical with succinic dehydrogenase. The continued investigation, however, did not bring conclusive arguments in favour of this suggestion. In the present investigation the relations between cytochrome *dh* and the rest of the system, particularly cytochrome *b*, will be elucidated and discussed.

The automatic recording spectrophotometer. The rapid recording technique introduced by the author (Lundegårdh 1951, 1953 a) enables the supervision of the

time course of increasing reduction, or reoxidation, of the single cytochromes. The absorption spectrum was recorded from a 15–17 mm. thick bundle of parallel packed pieces of roots (from the unbranched about 60 mm long end zone) which was introduced into a quartz tube provided with inlet and outlet for drainage and change of the medium during running exposure. The outgoing slit of a large quartz monochromator was focussed on the middle of the root bundle by means of a 45° metal mirror and a cylindrical quartz lens, the image forming a bright line of about the dimensions 0.2×4 mm. At the opposite side of the tube a 5 mm. diaphragm selects the transmitted light from the middle part of the root bundle. A high sensitive photo multiplier tube (E.M.I.), placed immediately below the diaphragm, records the intensity of the transmitted light on a Speedomax millivoltmeter. According to this arrangement stray light is completely avoided. Because the root bundle consists of several hundred pieces lying in close contact and the light beam meets the root surface perpendicularly no stray light is able to penetrate the root bundle. The recorded light must pass through thousands of single cells in which the cytochrome system is housing.

The wave length screw of the monochromator is automatically moved in intervals of 2 mμ. Each interval lasts 4 seconds or sufficiently long for attaining of a precise deflection of the millivoltmeter. If the instrument is fed by a 60 periods A.C. current the period is only 3.2 seconds. Alternatively a new Speedomax millivoltmeter with a deflection period of only 1–2 seconds was used. The quartz prism instrument is built for automatic recording of the reference beam, too. By means of a pneumatic device the object holder, carrying the recipient and the reference substance (mostly a layer of 12 pieces of Munkell filter paper nr. 3), is automatically moved in two positions alternatively exposing the reference beam and the sample to the photo tube. The two readings are printed by the Speedomax on a logarithmically graduated paper, from which then easily $\epsilon = \log \frac{I_0}{I_1}$ can be directly read as the distance between the two points; from these ϵ -values the *crude* spectrum is plotted.

The recording of fixed wave-length intervals is to be preferred to a continuous record if highest precision is to be attained, because all instruments suffer from inertia and an accurate continuous record of a spectrum hence presumes a too slowly moving wave-length screw. Because of the non-linear relation between the movement of the wave-length screw and the mμ values the construction of a prism instrument for continuous recording meets certain technical difficulties. Because of the higher spectral purity, particularly in the visible and ultra-red, prism instruments are, however, to be preferred for accurate measurements. In order to meet all needs in respect of rapid work, e.g. for the recording of the time course of reoxidation, a second instrument was built on the principle of a continuous movement of the wave-length screw.

In this instrument a Bausch & Lomb 500 mm. grating monochromator is combined with an electrically driven device which moves the wave-length screw one turn (= 100 mμ) in 50 seconds up to six minutes. The screw is turned synchronously with the paper chart of the high speed Speedomax instrument (period 1–2 seconds). With this instrument the reference beam has to be separately recorded, an arrangement which postulates a constantly burning light source (for the visible a 6 volt, 4.35 ampere, lamp on a large storage battery; for the ultraviolet a hydrogen lamp). A special device for eliminating the specific spectral sensitivity of the photo tube is omitted by this arrangement. The prism instrument, however, is provided with a

device eliminating the varying spectral sensitivity of the photo tube. Constructions have been tried which are based either on a mechanical or on an electronic principle. It was found that a mechanical device, consisting of an automatically adjustable diaphragm regulating the intensity of the entering light beam, combined with control readings of the reference intensity, secures highest accuracy.

If the sliding paper chart, on which the spectral curve is drawn by the grating monochromator, is provided with perpendicular lines marking each millimicron fairly accurate determinations of the wave-lengths may be performed. Another method is letting the instrument itself mark each millimicron on the chart. A simple arrangement for turning the continuous curve into a wave-line on which each top marks one millimicron is the use of an electrically steered shutter which produces flashes of slightly weakened light, one for each millimicron.

The O_2 -content of the recipient. The quartz recipient (see above) has a net volume of 4—6 ml. The dry weight of the root bundle is about 6 per cent of the fresh weight, or about 85 mg. The normal respiration of the bundle amounts to 20—25 $\mu\text{mol } O_2 \times h^{-1}$ if the O_2 -content of 1 lit. solution is 0.25—0.30 millimol (=saturation with air). The recipient thus consumes the oxygen dissolved in 100 ml. solution in one hour. The quantity of oxygen in 5 ml. aerated solution (=the volume of the recipient) amounts to about 2 μmol and is consumed in about 5 minutes if the flow of the solution is stopped. If the aerated solution before turning off the flow is rapidly exchanged for an O_2 -free solution (this takes only 1—2 seconds) the roots have consumed the quantity of oxygen present in the cells and intercellulars — comprising about 0.4 μmol — in less than one minute, provided that the respiration proceeds with undiminished speed to the end, which is, however, probably not the case. At an exponentially declining O_2 -consumption it will still take a few minutes before the last traces of oxygen are consumed. This is the main reason why the time course of reduction, after stopping the flow of the solution, is extended over several minutes. The reoxidation, after rapid turning on of the flow of an aerated solution, is considerably more rapid, because the molecules of oxygen are extremely rapidly moving in the tissue and a far lower number of them than those present before starting the reduction are needed for full oxidation of the small quantity of cytochrome present in the roots (on the absolute concentrations, see Lundegårdh 1953 a). Also other circumstances connected with the velocity of the single reactions in the whole chain are determining the rates of reduction and reoxidation (see Lundegårdh 1953 c and below).

The spectrum of cytochrome *b*

Whereas the α -band of what is now defined as a new cytochrome *dh* (Lundegårdh 1954 a) was observed at an early stage of these investigations (Lundegårdh 1952, 1953 a) the identification of the γ -band encountered difficulties. It was previously concluded (Lundegårdh 1953 a, p. 111) that the peak at c. 427.5 $m\mu$, observed in the difference spectrum ϵ anaerobic— ϵ aerobic, represented only cytochrome *b*. The spectrum was at that time recorded in intervals of 2.5 $m\mu$. Later records in intervals of 2 $m\mu$ and continuous records by means of the grating monochromator enabled a more detailed analysis. This resulted in the conclusion that the high γ -peak, observed in

the difference spectrum, represents not only the joined bands of *c*, *b*, and partly *a*, but also the γ -band of the new cytochrome *dh*. The partly opposite response of the cytochromes *c*, *b*, and *dh* to certain inhibitors, and their different apparent velocity constants at reduction (see below) then enabled the disclosure of previously rather puzzling examples of the appearance of more than three peaks of the compound γ -band.

The efforts of the biochemists to isolate the single cytochromes have hitherto been successful only in respect of the cytochromes *c*, *f* (Davenport and Hill 1952), and a component *b* forming a part of the lactodehydrogenase of bakers yeast (Appleby and Morton 1954). A comparatively pure preparation of cytochrome *b* from beef heart has been described by Hübscher, Kiese and Nicolas (1954). By the courtesy of Prof. Kiese the author had the opportunity to record the absorption spectrum of a preparation of comparatively pure cytochrome oxidase, sent by air mail. By the courtesy of Dr. R. Hill in Cambridge a pure sample of cytochrome *f* was brought for comparison in the authors spectrophotometers. Even if the bands of cytochrome *f* are very close to those of cytochrome *c* it is possible to analyse optically mixtures of the two substances. If the shape of the bands are accurately determined it is thus possible to analyse quantitatively a mixture of cytochromes provided that the concentration of one of the components is known.

For comparison with the roots absorption spectra were recorded from a Keilin-Hartree preparation of heart muscle and from a separation of cytochrome *b* according to the instructions given by Kiese and collaborators. Records were finally taken from baker's yeast in different stages of oxidation of the cytochrome system.

Cytochrome *dh* has not yet been biochemically isolated and the position of the bands can only be concluded from an optical analysis of the living roots. As peroxidase is always present in large quantities in the roots (Lundegårdh 1954 a, 1955 a) its optical behaviour has to be considered. Peroxidase is not reduced under anaerobiosis and it does consequently not interfere with the computation of the difference spectrum (ϵ anaerobic— ϵ aerobic), but it is easily combined with cyanide, fluoride, azide, and carbon monoxide (Keilin and Hartree 1951). Two separate publications are devoted to this question (Lundegårdh 1955). The bands of a number of cytochromes are given in table 1.

In living objects bands have been mentioned in the literature which may be interpreted as modifications of main groups, e.g. of *a*, *c*, and *b* respectively, but incisive spectrophotometrical investigations are very scanty and the work in this field is generally hampered by the fact that in the living cell not only active iron porphyrin enzymes are operating but also processes are going on, in which these enzymes are synthetized or decomposed (cf.

Table 1. *Absorption bands of some cytochromes (m μ).*

Cytochromes	α		β		γ	
	ox	red	ox	red	ox	red
<i>c</i>	—	550	—	520	406	416
<i>f</i>	—	554	—	526	?	422
<i>b</i> ₂ (Appleby and Morton 1954)	—	557	—	528	413	424
<i>b</i> (Kiese et al.)	556	562/563	525	530	420	431/432
<i>dh</i>	—	571	—	540	—	424
<i>a</i>	—	603/604	—	?	420	443

Ephrussy and Slominsky 1950, Chin 1950 on baker's yeast.) Also adaptive transformations of cytochromes are obviously proceeding (Lundegårdh 1954 c). Only a close observation of the spectral behaviour of the iron porphyrins in the objects, e.g. by the rapid and sensitive technique introduced by the author, is here able to disclose the eventual enzymatic properties of the substances in question. Unfortunately the chemical side of the enzymatic processes going on in living tissues is considerably more difficult to unveil because produced substances are at least temporarily withheld from the medium and added substances are frequently not absorbed as willingly as could be wished. The excellent property of most oxidation-reduction enzymes (cytochromes, flavoproteins, coenzymes) to reveal their state of oxidation-reduction in the absorption spectrum can at present only under favourable conditions be completed by a similar supervision of the reacting chemical components. Measurements of the respiration simultaneously with the spectrophotometric work may, however, be made (Lundegårdh 1954 c).

The cytochrome *b* of wheat roots has an absorption spectrum very similar to the corresponding enzyme of the heart muscle, whereas cytochrome *b* in baker's yeast shows a somewhat lower position of the α -band (figures 1—3). It may be remarked, however, that reduction with dithionite slightly moves the α -band and the β -band to an about 1 m μ lower value. Baker's yeast is abundantly provided with cytochrome oxidase (figure 2). The relation between *a* : *c* : *b* in roots is about 1 : 2 : 4, in baker's yeast about 1 : 2 : 2. The dominating concentration of cytochrome *b* in roots tallies with its activity as an oxidase in the «ground respiration», as shown in a previous communication (Lundegårdh 1955 b).

The statement that the γ -band of cytochrome *b* in roots has its position very close to the corresponding enzyme in the heart muscle, viz. 431—432 m μ (see Hübscher, Kiese and Nicolas 1954 and figures 1 and 3), shows that the peak at 427—428 m μ in the spectrum of reduced roots (Lundegårdh 1953 a and fig. 3) results from the combination with a band of somewhat lower

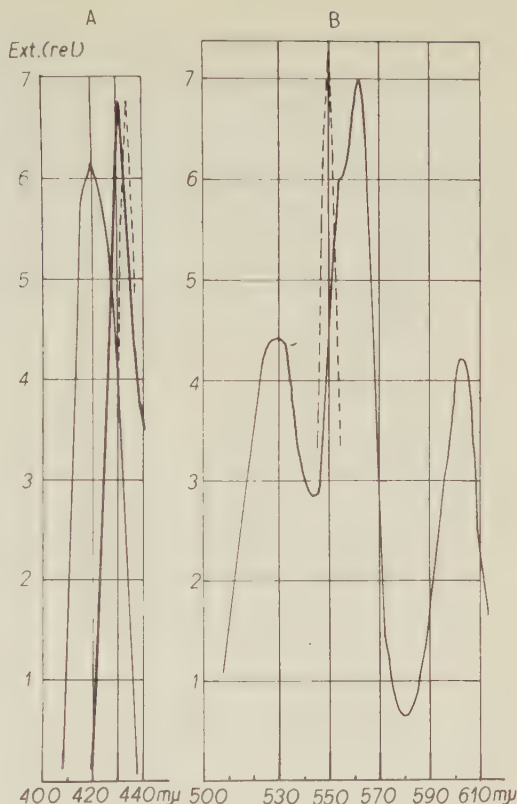


Figure 1. Spectrograms of a Keilin-Hartree preparation from cow heart, diluted with 0.05 M phosphate buffer, pH 8.2. A: bands in the Soret region of the oxidized preparation (to the left, peak at 420 mμ) and after addition of 0.01 M dithionite (to the right, peak at 431 mμ). The bands predominantly represent cytochrome *b*, as may be seen from the reduced bands in the α - and β -region (B), where *b* strongly dominates over cytochrome *c*. At 604 mμ a strong band of cytochrome oxidase. The dotted band in A is the difference curve ϵ reduced — ϵ oxidized, the dotted band in B is a record of the reduced α -band of pure cytochrome *c* (for control of the wavelengths).

value. If the observed γ -band were composed only by the cytochromes *c* and *b* it would show a twin peak at c. 418 mμ (=the peak of *c* in the difference spectrum) and at c. 430—433 mμ (the corresponding peak of *b*), the latter peak being considerably higher. It was namely shown (Lundegårdh 1953 a, p. 103) that two bands lying at a distance of more than 10 mμ (in the present case 13—15 mμ) appear as two peaks from a joined base. Bands lying at a distance of only c. 5 mμ are always fused to a single peak. The observed shape of the γ -band thus indicates the presence of a third band between 418 and 431 mμ. This is now defined as the γ -band of cytochrome *dh* and its position is very near to 424 mμ.

Figure 3 shows the optical analysis and reconstruction of the observed joined γ -band from roots which were reduced by 0.01 m dithionite in 0.02 m phosphate buffer at pH 5.9. The single bands of *c*, *b*, and *dh* are calculated from the observed separately appearing α -bands at 550, 563, and 571 mμ respectively, assuming the relation $\frac{\gamma}{\alpha} = 3$ for *c* and *b*, and $= 2$ for *dh*.

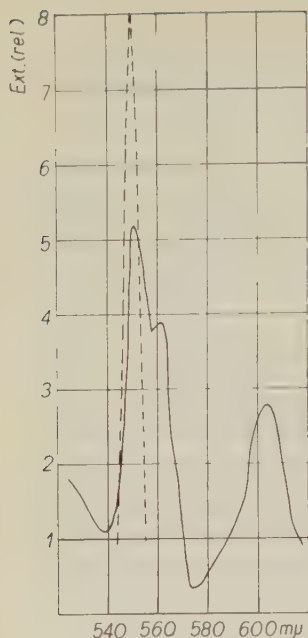
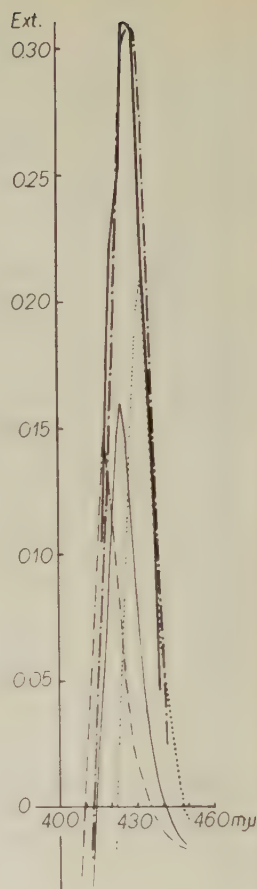


Figure 2. Spectrogram of a suspension of 15 per cent baker's yeast in phosphate buffer. Length of the cuvette 17 mm. The peak representing cytochrome *c* is slightly to the right of that of a pure preparation (dotted curve). The peak of cytochrome *b* is 1—2 mμ to the left of 563 mμ. The peak of cytochrome oxidase coincides with that of the heart muscle preparation.

Figure 3. Difference spectrum of a 17 mm. thick bundle of wheat roots (ϵ reduced in 0.01 *M* dithionite + 0.02 phosphate at pH 5.7— ϵ aerobic phosphate). ——— Observed curve. ——— Calculated γ -band from ——— cytochrome *c*, cytochrome *b* and ——— cytochrome *dh*. The principles of the calculation are given in the text.



As shown in figure 3 the calculated fused band coincides fairly well with the observed band. The value $\frac{\gamma}{\alpha} = 3$ has been calculated from direct measurements with regard to the difference spectrum of *c* and *b*. In the crude spectrum of the reduced cytochromes the relation amounts to about twice that figure. As to cytochrome *dh* no calculation is yet available, because the γ -band of the oxidized cytochrome is apparently confounded with the bands of *a* and *c*. If the γ -band of *dh* has a similar shape as that of *c* and *b* the lower value of $\frac{\gamma}{\alpha}$ points to a rather small distance between the γ -bands of the oxidized and reduced cytochrome. Or the relation $\frac{\gamma}{\alpha}$ in the reduced spectrum is lower than what is normal for other cytochromes. It is to be observed that the spectrum of *dh* deviates from that of other cytochromes in the comparatively low mμ value of the γ -band (see table 1).

The time-course of reduction and oxidation

As previously shown (Lundegårdh 1953 c) the time-course of reduction (=change from aerated to O₂-free) is considerably more extended than that of reoxidation (=change from O₂-free to aerated). Besides of the interfering O₂-content of the tissue (see above) the chief reason for this is obviously the fact that the reduction starts from the normally existing predominantly oxidized cytochromes (65–85 per cent oxidized) and is regulated by the moderate activity of the dehydrogenase systems. Of the two processes at the bottom of the potential ladder, viz.

1. $A \cdot H_2 + \text{Deh.} \rightleftharpoons A + \text{Deh.} \cdot H_2$
2. $\frac{1}{2} \text{Deh.} \cdot H_2 + \text{Cyt.}_{\text{ox}} \rightleftharpoons \text{Deh.} + H^+ + \text{Cyt.}_{\text{red}}$

the first one acts as a brake on the second, which, as the following electron transferences, proceeds very rapidly. The high velocity of the electron transference is shown by the high speed of reoxidation, which of course runs at top speed just in the beginning when all cytochromes are reduced. It could be calculated that the start velocity of reoxidation amounts to a turnover per minute of more than 20,000 (Lundegårdh 1953 c). From the fact that the reoxidation of a completely reduced cytochrome system does not exceed the level of oxidation maintained in the normal life of the roots it can be concluded that the dehydrogenase systems remain intact and continuously retard the reoxidation. The actual velocity of the electron transference is then probably at least hundred times faster than observed, a fact illustrating the close structural intimacy between the components of the multiplex enzyme system. The fact that the cytochromes in the roots are normally predominantly oxidized is a direct illustration of the relatively slow working dehydrogenases. In baker's yeast the balance is normally displaced to the side of predominant reduction of the cytochromes (see figure 2).

The time-course of *reoxidation* of the different cytochromes of wheat roots may be slightly varying but on an average proceeds in the order $dh > b \geq \begin{matrix} c \\ a \end{matrix}$. It was recently shown (Lundegårdh 1955 b) that cytochrome *b* in wheat roots acts as an oxidase with an effective power of about 10–15 per cent of that of cytochrome oxidase. No appreciable autoxidation of cytochrome *dh* could be detected, but the experimental evidence points in favour of a redox equilibrium between *b* and *dh*, the potential state of the former being more positive.

In the rather complicated balance created by the fact that both *a* and *b* emit electrons to oxygen the order of reoxidation will be regulated by the apparent velocity constants. Due attention must here, of course, be paid to

the relative molar concentration of the enzymes (see Lundegårdh 1953 c). Attention must be paid, too, to the coexistence of dehydrogenases acting as retarding factors. The available experimental evidence (see Lundegårdh 1953 a, 1954 c) points in direction of cytochrome *b* being linked to succinic dehydrogenase and, in close cooperation with *c*, linked also to a second dehydrogenase system, which is operating by means of flavoprotein (Slater factor [Slater 1950]; »flavoprotein junction» [Lundegårdh 1954 c]) and probably coenzyme. As the two cytochromes *c* and *b* are joined in a reversible electron transference the localization of the junctions to the dehydrogenases is an intricate problem. The junctions to dehydrogenases act, however, retarding on the reoxidation of both *c* and *b*, a fact explaining the leading of *dh* at reoxidation. Cytochrome *dh* is probably not directly linked to succinodehydrogenase (see below). Its behaviour bears witness of a comparatively slow reduction.

According to varying growth conditions the molar relation $\frac{\text{cytochrome } b}{\text{cytochrome } c}$ is varying in different sets of plants, a circumstance reflected in the time order of reduction and reoxidation. Two experiments gave the following results:

$\frac{\text{Cytochrome } b}{\text{Cytochrome } c} = \dots\dots\dots$	0.95	2.7
Half time of reoxidation of cytochrome <i>c</i>	< 20 seconds	110 seconds

This result is in accord with what might be expected from the difference in the potential state of the two cytochromes: *c* has a predominant tendency to be reduced by *b*. It was observed that a comparatively high concentration of cytochrome *b* accelerates the reoxidation of cytochrome *dh*. Two experiments may be mentioned here:

conc. Cytochrome <i>b</i> (relative)	200	100
Velocity of reoxidation of cytochrome <i>dh</i> (relative)	150	100

An example of the time order $dh > b$ at reoxidation is given in figure 4. Two records are reproduced, one in the 40—70 seconds after the change of the medium from anaerobic to aerobic, the other in the period 150—200 seconds. The difference curve between these two gives an approximately pure γ -band of cytochrome *b*. The peak of the fused bands of *c*, *dh*, and *b* (see above) is accordingly moved from almost exactly 424 m μ , representing the dominating γ -band of *dh*, to an intermediate position between 424 and 431 m μ , the latter figure representing the top of the γ -band of cytochrome *b*.

At reduction the time order is the reverse of that of reoxidation, viz. $a > c > b > dh$. As no electrons can be emitted from the cytochrome oxidase

Figure 4. Difference curve (ϵ aerobic— ϵ anaerobic) illustrating the reoxidation of a root bundle. Two stages of the time course, one after 40—70, the other after 150—200 seconds. Below, the difference between the two curves, coinciding with the γ -band of cytochrome *b*.

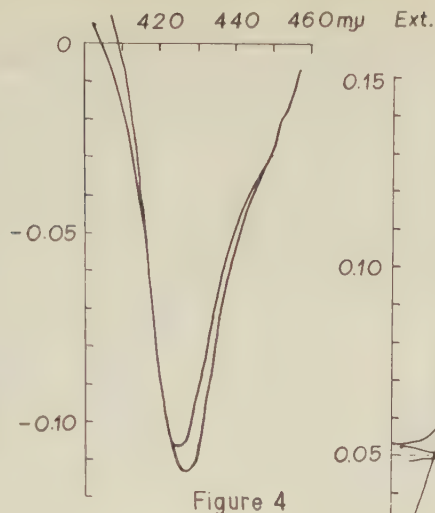
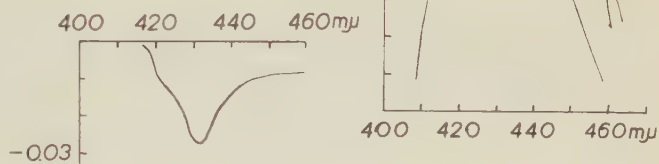


Figure 5. Time course of increasing reduction in O_2 -free succinate (0.05 *M* at pH 4.3). Time interval from the start: 1. 60 sec., 2. 240 sec., 3. 7 min., 4. 13 min., 5. 34 min.



in the absence of its specific acceptor, oxygen, it is extremely rapidly reduced by cytochrome *c*. Cytochrome *c* is in its turn nearly as rapidly reduced by cytochrome *b* and/or the flavoprotein junction. Owing to the complicated optical situation in the γ -region (cf. Lundegårdh 1955 a and above), and possibly also to complex reactions (Lundegårdh 1953 d), the response of cytochrome *c* on a change in the oxidation-reduction state is preferably observed on the α -band at 550 $m\mu$. This band appears almost instantaneously after the removal of the oxygen (figure 2 in Lundegårdh 1953 c). The rapid appearance of the γ -band at 443 $m\mu$ of cytochrome oxidase is shown in fig. 5. It is, however, fully developed only after 240 seconds, probably owing to the slow disappearance of the last traces of oxygen in the root tissue (see above). It may be added here that the actual concentration of oxygen in an aerated tissue is not known and that the possibility cannot be dismissed that certain proteins act as oxygen carriers. As shown by the extremely rapid start of reoxidation the non-metabolic transport of oxygen through the root tissue is not a limiting factor, provided that the concentration of oxygen is not appreciably falling below the exchange equilibrium with air (see above).

Figure 6 gives still another example of the separation in time of the cytochromes *b* and *dh*. A difference curve, calculated from the situation after

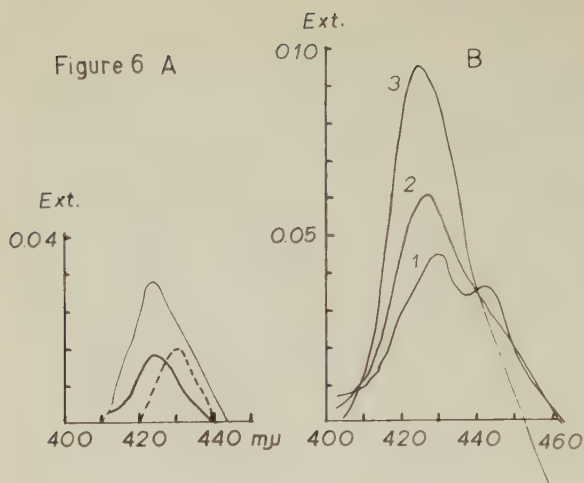


Figure 6. Time course of reduction in O_2 -free 0.05 M phosphate, pH 4.35. B. From the start: 1. 2 1/2 min., 2. 5 min., 3. 10 min. A. Difference curves between 120 and 60 sec. (---), representing cytochrome *b*, between 300 and 120 sec. (—), representing *dh*, and between 10 minutes and 5 minutes (—), also *dh*.

300 and 120 seconds respectively, shows a band at 425 mμ. (= *dh*), whereas the difference between 120 and 60 seconds gives a band at 430—431 mμ (= *b*). The time order at reduction, viz. *b* > *dh*, conveys a displacement of the peak of the joined bands of *b* and *dh* from c. 431 mμ at the start to c. 425—426 mμ after 20 minutes (see figure 6 B).

Optical separation of the steady states of cytochromes *b* and *dh* under the influence of various inhibitors

As expected succinate (0.1 M at pH 3.6; on the inverse relation between dissociation and absorption, see Lundegårdh 1949) moves the oxidation-reduction balance of all cytochromes in the direction of increased reduction. Probably because of the rapid synthesis of succinate in the normal metabolism of the root the increase is not large, from 15 to 44 per cent above the level maintained in an aerated salt solution. Succinate does not affect any single step, but accelerates the reduction of the total chain.

Fumarate (0.1 M at pH 3.6) acts opposite to succinate, viz. displaces the balance in direction of a considerably increased oxidation of the system. This was previously (Lundegårdh 1953 a) interpreted as a shifting of the equilibrium $\text{succinate} \rightleftharpoons \text{fumarate} + H_2$ from the right to the left and concomittantly a reversal of the stream of electrons, resulting in a decreased reduction of the cytochromes even under anaerobical conditions. But in addition to that fumarate obviously acts more specifically on different points of the chain. It was observed that the level of oxidation in fumarate is $c > b > dh$, indicating a practically stopped emittance of electrons from *b*

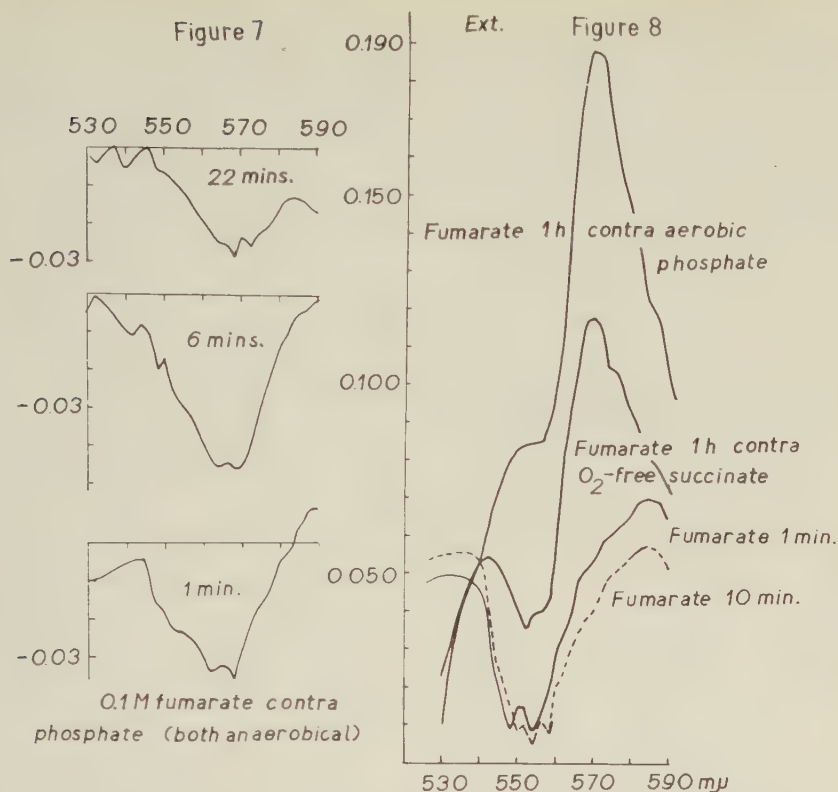


Figure 7. Time course of the anaerobic oxidation of the cytochromes *b*, *c* and *dh* in 0.1 M fumarate contra phosphate.

Figure 8. Time course of the decreasing oxidation and increasing reduction at prolonged stay in fumarate. Very strong reduction of cytochrome *dh*, even in comparison to succinate.

to *c*. The observation of the time course (see figures 7 and 8) furthermore shows a gradual shifting over of cytochrome *dh* from a slightly over-oxidized situation to strong reduction. After a prolonged stay in O₂-free fumarate cytochrome *dh* is finally reduced about 2.6—2.7 times more than at anaerobiosis in the absence of fumarate (figure 8).

From these results two important conclusions can be drawn: (1) that cytochrome *dh* is never reduced to more than about 40 per cent under anaerobic conditions, and (2) that fumarate finally reverses the equilibrium $b \xrightleftharpoons[c]{e} dh$. This reversal is not observed in succinate but it is even more accentuated if ATP is added to the fumarate (figure 9). This result will be discussed in the following section.

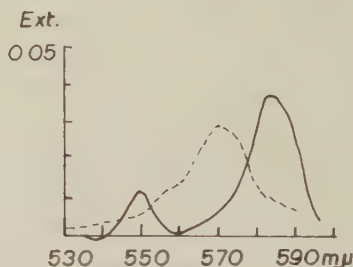
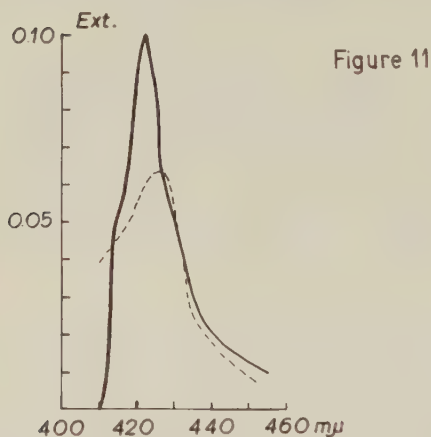
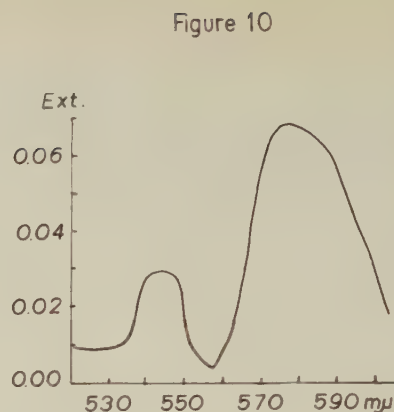
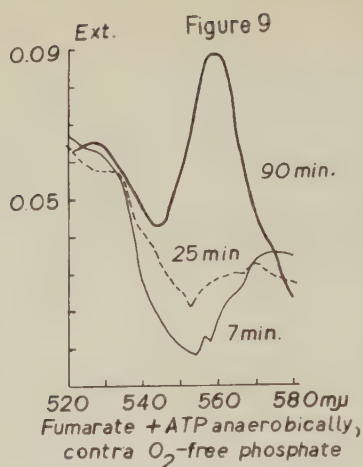


Figure 9. The final reduction of cytochrome *dh* in fumarate + ATP.

Figure 10. Difference spectrum ϵ 0.1 M malonate + HCN— ϵ phosphate + HCN.

Figure 11. Difference spectra — ϵ O₂-free 0.05 M KCl— ϵ O₂-free 10 per cent urethane, showing spectrum of cytochrome *dh*. - - - ϵ O₂-free urethane— ϵ aerated urethane, showing spectrum of cytochrome *b*.

Malonate (0.1 M at pH 3.4) acts much in the same way as fumarate, viz. causes oxidation of *b* and reduction of *dh*, thus a reversal of the normal state. At simultaneous inhibition with cyanide malonate thus produces a difference spectrum of cytochrome *dh* (figure 10), but the bands are extended towards red, a fact probably indicating a partial decomposition. Malonate severely affects the microscopic configuration of the protoplasm (Lundegårdh 1949).

Urethane (10 per cent solution). According to current opinions (Keilin and

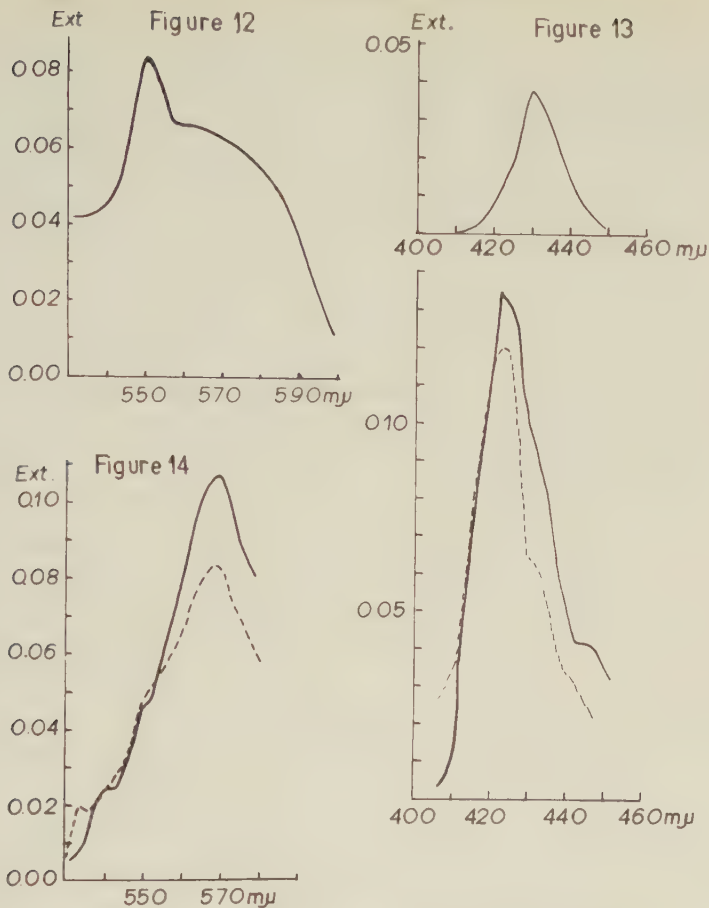


Figure 12. Difference spectrum ϵ (O₂-free urethane)— ϵ aerated urethane, showing strong oxidation of cytochrome *c*.

Figure 13. Reduction after pretreatment — 10⁻⁶ M and - - - - 10⁻⁵ M dinitrophenol (DNP). Above the difference between these curves, coinciding with the γ -band of cytochrome *b*.

Figure 14. — reduction in distilled water. - - - - reduction in a solution of 0.1 mg Antimycin A in 100 ml aqua.

Hartree 1939; cf. Lundegårdh 1953 a) urethane inhibits the electron transference from *b* to *c*, viz. the oxidation of *b*. If 10 per cent urethane is applied aerobically an increased reduction of *b* and *dh* can actually be observed. This group is accordingly electronically partly isolated from the *a*—*c* group. If, however, urethane is applied anaerobically and the medium is then changed to an aerated solution of urethane the spectrophotometer shows a partial reoxidation of cytochrome *b*, whereas *dh* remains reduced. Urethane

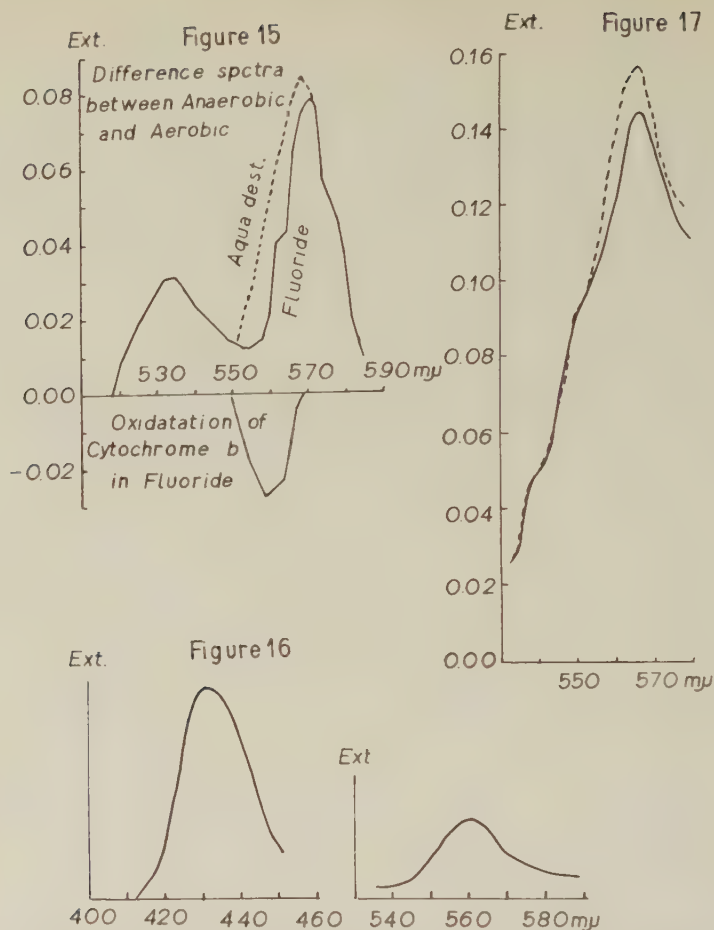


Figure 15. The oxidation of cytochrome *b* in the presence of fluoride (0.03 *M* at pH 3.3). — reduction in distilled water, - - - - reduction in fluoride. Below the difference between the curves, coinciding with cytochrome *b*.

Figure 16. Spectrum of cytochrome *b*, appearing as the difference in reduction without and with fluoride.

Figure 17. - - - - reduction in phosphate solution. — reduction in a 0.003 *M* solution of ATP.

retards the electron transference from *b* to *c* only to about 50 per cent, but the electron transference from *dh* to *b* considerably more. As a consequence of that a comparatively pure spectrum of *dh* can be computed from ϵ anaerobic urethane— ϵ aerobic urethane (fig. 11). The pronounced oxidation of cytochrome *c*, as compared with *b*, is shown in figure 12.

Dinitrophenol (DNP) and *phenylthiourea* exert effects similar to urethane on the balance between *b* and *dh*, only still more pronounced. As shown in figure 13 a pure γ -band of cytochrome *b* is obtained as the difference between spectra computed after treatment with 10^{-5} and 10^{-6} *M* DNP respectively. The right side of the combined γ -band gradually disappears, as the concentration of DNP is increased, whereas the left side of the band, representing *dh*+*c*, remains intact.

2.4 *D* (10^{-5} *M*) partially inhibits the reduction of cytochromes *b* and *dh* (to about 40 per cent), whereas *c* behaves more independently. Similarly to urethane, DNP, and phenylthiourea this inhibitor thus retards the transference of electrons between the cytochromes *b* and *c*. As a consequence of that cytochrome *c* is widely oxidized at reoxidation, whereas *b* and *dh* remain 17–20 per cent more reduced than without the inhibitor.

Antimycin A. This antibiotics, of which a sample was kindly sent by Prof. F. M. Strong at Madison, Wisconsin, retards the transference of electrons from *b* to *c* (cf. Potter and Reif 1952) if given in a concentration of 0.1 mg in 100 cc (figure 14). A concentration of 1 mg in 100 cc retards the reduction of the system, too.

Fluoride. As shown in a previous communication (Lundegårdh 1953 a) fluoride (0.03 *M* at pH 3.3) almost completely uncouples cytochrome *b* from the dehydrogenases and from *dh*, thus promoting the complete oxidation of the former. In contrast to that fluoride has only little effect on the oxidation-reduction balance of cytochrome *dh*. This inhibitor may thus be used for the optical isolation of cytochrome *b* (see figures 15 and 16).

The effect of ATP

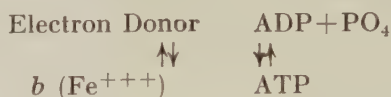
It was mentioned above that the addition of ATP to fumarate enhances the anaerobic oxidation of cytochrome *b* and partly cytochrome *c* (fig. 10). If the joined γ -band of *b* and *dh* is measured in O_2 -free inorganic phosphate and, after a rapid change of the medium, in O_2 -free 0.003 *M* ATP a distinct lowering of the peak is observed (figure 17), a fact indicating a reversal of the stream of electrons from the dehydrogenase systems. The oxidizing effect of ATP is observed under aerobical conditions, too. Inorganic phosphate, on the contrary, rises the level of reduction, as compared with a dilute solution of KCl (see table 2).

The observed facts are in agreement with the idea that the transference of electrons from a donor to cytochrome *b* supplies the energy for the syn-

Table 2. *The effect of ATP and inorganic phosphate on the steady state of the cytochromes.*

Medium	Per cent oxidized cytochrome			
	Conditions	Cyt. <i>c</i>	Cyt. <i>b</i>	Cyt. <i>dh</i>
0.05 <i>M</i> KCl pH 5.7	aerobic	65	85	90
0.5 <i>M</i> Phosphate pH 6.0	»	—	67	69
2.4 mg ATP in 100 ml aqua	»	80	c. 100	c. 100
0.05 <i>M</i> KCl pH 5.7	anaerobic	0	0	0
2.4 mg ATP in 100 ml aqua	»	30	35	28

thesis of ATP, the coupled reactions being reversible tentatively according to the following scheme:



At a sufficient supply of PO_4 (and ADP) and donors and acceptors of electrons, viz. at continuously working respiration, the processes are proceeding in the downward direction. An accumulation of ATP (or any equivalent \sim ph compound) slows down the electron transference and may under anaerobical conditions reverse the equilibrium, resulting in a considerable oxidation of cytochrome *b*.

The relations between the two cytochromes *b* and *dh* in connection with the \sim ph metabolism are in accord with the conception of *dh* as one of the electron donors of *b*. The fact that cytochrome *dh* finally is turned over into a state of strong reduction under the influence of ATP (and fumarate), simultaneously with cytochrome *b* remaining more oxidized, may be interpreted as the reversal of an electron donor function, according to the hypothesis.

The observed anaerobic oxidation of cytochrome *b* by ATP forms a direct evidence against the transference $b \xrightleftharpoons[c]{e} c$ being involved in the \sim ph balance, because such a coupling would lead to a reduction of *b* at a reversal of the equilibrium, hence the opposite to what is observed. The \sim ph equilibrium is thus linked to the dehydrogenase side of cytochrome *b*, with cytochrome *dh* and succinodehydrogenase as electron donors (see below).

The opposite effects of inorganic phosphate and ATP on the \sim ph balance are in accord with the effect of DNP, which is known as an inhibitor of phosphorylation (see above). A weakening of the phosphorylation, viz. the synthesis of \sim ph must, according to our scheme, also retard the electron transference from the dehydrogenases to cytochrome *b*, with the observed increase in oxidation as the visible consequence. As shown in the previous

section a number of substances more or less slow down the velocity of reduction of cytochrome *b*. This point is in fact one of the most sensitive ones in the aerobic metabolism and also one of the most important for the life of the cell, because it obviously contributes to the regulation of the \sim ph metabolism. This may be the explanation of the inhibiting effect on the growth which most of these inhibitors of the electron transference to cytochrome *b* are exerting. Also HCN, besides of its inactivation of the cytochrome oxidase, has a tendency to reverse the electron transference between the dehydrogenases and cytochrome *b* (Lundegårdh 1955 b).

Discussion

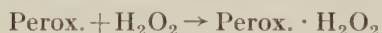
The experimental observations support the conclusion that cytochrome *dh* participates in the complicated system of reactions conducted by the cytochrome system. It was previously supposed (Lundegårdh 1953 a) that *dh* might possibly be identical with the succinic dehydrogenase. An argument against this possibility is the fact that no trace of cytochrome *dh* can be detected in Keilin-Hartree preparations of heart muscle, the oxidizing effect of succinate on which is well known (cf. figures 1 and 3). The available facts are more in accord with the idea of a multiplex linkage between cytochrome *b* and factors acting as dehydrogenases, one of which represents succinodehydrogenase, and that cytochrome *dh* in some way is coordinated with *b* and reinforces or widens its field of activity. This widening of the scope of cytochrome *b* is probably devoted to processes characterizing certain cells of higher plants, as distinguished from animals and microorganisms.

Besides of succinodehydrogenase the root cells are provided with a second donor of electrons and protons which has been identified with a flavoprotein, the concentration and state of oxidation-reduction of which coincides with the »ground respiration» (Lundegårdh 1954 c). Because the cytochromes *b* and *c* are joined in a reversible equilibrium (Lundegårdh 1953 a) it is difficult to ascertain if the »flavoprotein junction» is connected to one of them or to both. Appleby and Morton (1954) recently discovered a close molecular linkage between flavoprotein and a cytochrome *b* both together composing the lactodehydrogenase of baker's yeast. Per analogiam the possibility has to be investigated if a close cooperation between flavoprotein and cytochrome *b* acts as succinodehydrogenase, but such a possibility does not exclude the existence of a flavoprotein junction leading over to other donors of hydrogen than succinate, too. The experiments with fumarate may be interpreted according to the following scheme ($D=H$ -acceptor from succinate):



The fact that the initially pronounced oxidation of *b* in fumarate (=the reversal of the process) slowly decreases (cf. figures 7—9) may depend upon a final cessation of the reversal because of accumulated non-oxidized succinate or upon a secondary effect. The flavoprotein junction is obviously able to furnish electrons to cytochrome *b* after the uncoupling of the group *a*—*c* in cyanide, because *b* is autoxidizable and unaffected by HCN (Lundegårdh 1955 b). This means, however, a considerable turning down of all processes linked to cytochrome *b* as an acceptor of electrons because the capacity of *b* as an oxidase is only 10—15 per cent of that of *a*. The existence of cytochrome *dh* in organs capable of a ground respiration may perhaps be interpreted as a reserve donor of electrons. The very slow reduction of *dh* under anaerobical conditions indicates some such property of *dh*. This enzyme may possibly over-take electrons and protons also from circulating reducing substances, as ascorbic acid, etc.

Cytochrome *dh* behaves in several respects as a true cytochrome but it deviates from the other members of this group by its unwillingness to undergo complete reduction. Of other iron porphyrin enzymes peroxidase and catalase are not reduced at all under anaerobic conditions. Cytochrome *dh* is here reduced to about 40 per cent. This fact actualizes the possibility that *dh* may have an intermediate position between peroxidase and cytochromes a circumstance which would also explain the »anormal» position of the γ -bands. Peroxidase is figured to act according to the following scheme (cf. Chance 1951):



The peroxidase-hydroperoxide complex is here thought to act as a hydrogen acceptor. An iron porphyrin enzyme acting as hydrogen carrier is not known, but it is difficult to understand why a peroxide complex could act as H-acceptor if the hydrogen was not attracted by some group in the protein. An iron porphyrin enzyme directly acting as hydrogen carrier would probably have its iron in the trivalent stage. It could act, too, as the first acceptor of electrons at the dissociation of H in $\text{H}^+ + e$ if the electron was immediately transferred to cytochrome *b*, viz. if the potential states of the H-carrier was appreciably lower than that of *b*, so that the enzyme remained in a predominantly oxidized state. The spectral properties of cytochrome *dh* are not incompatible with this hypothesis but further research work is needed before more weighty conclusions can be drawn.

The involvement of cytochrome *b* in an reversible \sim ph balance may certainly contribute to the retardation of its anaerobical reduction. The opinion advanced by Chance (1953) as to cytochrome *b* according to him not lying in

the main path of electron transference from dehydrogenases to oxygen is not convincing. The more incisive study of both reduction and reoxidation, performed by the author, revealed the extremely rapid electron transference between *all* cytochromes (*dh*, *b*, *c*, *a*), recognizable at reoxidation. The slower response of cytochrome *b*, as compared with *c*, at reduction under anaerobiosis reflects the moderate speed of the start reaction (p. 149) and the buffer action of the \sim ph balance. This buffer action is of considerable interest from the viewpoint of metabolic economy of the cell. As long as supplies of high energy phosphate (ATP or its equivalents) are available cytochrome *b* may be anaerobically reoxidized and in its turn accept electrons from systems of lower potential stage. This phenomenon thus enables a limited continuation of the tricarboxylic cycle and of intramolecular oxidations conducted by coenzymes without the necessity for the formation of alcohol or lactate as final hydrogen acceptors. The consumption of high energy phosphate via cytochrome *b* retards the transition from aerobic respiration to pure glycolysis. This is probably very important because one of the products of glycolysis, alcohol, is a dangerous poison to the root tissue. In connexion with the participation of parts of the cytochrome system in an intermediate anaerobic respiration an observation may be mentioned which the author made several years ago. In pursuing the variation of the »glucose quotient», viz. the relation

$\frac{\text{consumed glucose}}{\text{formed CO}_2} \times 6$ it was found (Lundegårdh 1950, p. 337) that under

anaerobiosis the value amounts to 3 in distilled water as medium, as may be expected from the switching on of alcohol formation. If the medium is changed to a salt solution the quotient, however, sinks down to 2 or even lower. This fact can only be explained by the start of some process implying a more economic consumption of glucose, viz. more of the kind of combustion. It has been shown (Lundegårdh 1951, 1953 a, b) that salt anions act as coenzymes in the electron transference between the cytochromes. Without movable anions no activity of the cytochromes. The observations of the glucose quotient display a conclusive argument in favour of the assumption that part of the cytochrome system, with high energy phosphate as fuel, saves the root tissue from rapid poisoning by products of glycolysis.

Conclusions and summary

A new cytochrome, *dh*, has been identified in roots of wheat and other cereals. Its spectrum and behaviour under varying experimental conditions has been compared with the spectrum of cytochrome *b*. The new cytochrome is related to cytochrome *b* and to peroxidase in respect of its prosthetic group,

which is protohaemin (Lundegårdh 1954 a). Cytochrome *dh* is under normal aerobic conditions strongly oxidized and it is only incompletely (to about 40 per cent) reduced under anaerobiosis. Its response to reduction and reoxidation, to a number of inhibitors, and to fumarate and ATP indicates a reversible electron transference between the cytochromes *b* and *dh*.

Cytochrome *b* as an electron acceptor is closely linked to a reversible ~ph balance, which in the absence of oxygen promotes a partial oxidation of this cytochrome. The crosslinkage between cytochrome *b* and its electron donors (dehydrogenases, cytochrome *dh*) on one side and the reversible high energy phosphorylation on the other side acts as a buffer system*delaying the reduction of cytochrome *b* under anaerobiosis. Another brake on the cytochrome system is the start reaction of dehydrogenation. The electron transference between all cytochromes is extremely fast and has no limiting influence. The apparent retardation, by a number of inhibitors, of the electron transference proceeding on the dehydrogenase side of cytochrome *b*, which is the most sensitive point of the system, is probably chiefly caused by influences on the phosphorylation, but may also imply structural changes of the protein carrier or the structural organization of the system.

The possible properties of cytochrome *dh* are discussed.

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References

- Appleby, C. A. & Morton, R. K.: Crystalline Cytochrome *b*₂ and Lactic Dehydrogenase of Yeast. — *Nature* 173: 749. 1954.
- Chance, B.: The Iron-Containing Enzymes. C. The Enzyme-Substrate Compounds and Mechanism of Action of the Hydroperoxidases. — *The Enzymes* (Sumner, Myrbäck) Vol. 2, Part 1. 428. 1951.
- Chance, B.: Spectra and Reaction Kinetics of Respiratory Pigments of Homogenized and Intact Cells. — *Nature* 169: 215. 1953.
- Davenport, H. E. & Hill, R.: The Preparation and Some Properties of Cytochrome *f*. — *Proc. Roy. Soc. B* 139: 327. 1952.
- Hübscher, G., Kiese, M. & Nicolas, R.: Untersuchungen über Cytochrome. III. Cytochrom *b* aus Rinderherzen. — *Biochem. Zeitschr.* 325: 223. 1954.
- Keilin, D. & Hartree, E. F.: Cytochrome and Cytochrome Oxidase. — *Proc. Roy. Soc. B* 127: 167. 1939.
- — Purification of Horse-Radish Peroxidase and Comparison of its Properties with those of Catalase and Methaemoglobin. — *Biochem. Journ.* 49: 88. 1951.
- Lundegårdh, H.: Growth, Bleeding and Salt Absorption of Wheat Roots as Influenced by Substances which Interfere with Glycolysis and aerobic Respiration. — *Ann. Agric. Coll. Sweden* 16: 339. 1949.
- *Lärobok i Växtfysiologi*. — Stockholm 1950.
- Spectroscopic Evidence of the Participation of the Cytochrome-Cytochrome Oxidase System in the Active Transport of Salts. — *Ark. f. Kemi* 3: 69. 1951.

- Properties of the Cytochrome System of Wheat Roots. — Ark. f. Kemi 5: 97. 1953 a.
 - Controlling Effects of Salts on the Activity of the Cytochrome Oxidase. — Nature 171: 477. 1953 b.
 - Reaction Kinetics of the Cytochrome System. — Nature 171: 521. 1953 c.
 - Complex Reactions of Cytochrome *c*. — Nature 172: 303. 1953 d.
 - A New Cytochrome in Living Roots. — Nature 173: 939. 1954 a.
 - On the Oxidation of Cytochrome *f* by Light. — Physiol. Plant. 7: 375. 1954 b.
 - Enzyme Systems Conducting the Aerobic Respiration of Roots of Wheat and Rye. — Ark. f. Kemi 7: 451. 1954 c.
 - Spectrophotometrical Determination of Peroxidase in Living Roots. — Physiol. Plant. 8: 84. 1955 a.
 - On partial Oxidation of the Cytochrome System in the Presence of Cyanide. — Physiol. Plant. 8: 95. 1955 b.
- Potter, Van & Reif, A. E.: Inhibition of an Electron Transport Component by Antimycin A. — J. Biol. Chem. 194: 287. 1952.
- Slater, E. C.: Components of the Dihydrocozymase Oxidase System. — Biochem. Journ. 46: 484. 1950.

Corrigenda. The μ values are in figure 9 ten units below and in figure 11 (to the right) ten units above the correct ones.

Further Experiments on the Response of Decotylised Pea Seedlings to Arginine

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Introduction

In two recently published papers (Fries 1953, 1954) it was demonstrated that arginine, glycine and adenine when added to the agar medium of decotylised pea seedlings cultivated in darkness, promoted the growth of the plants, particularly that of the root. This was explained as the result of a less efficient biosynthesis of these metabolites in the etiolated seedlings.

The growth-promoting effect of the substances in question manifested itself when the experimental conditions were changed in various ways, e.g. as regards the amount of available nitrogen in the medium, hydrogen ion concentration, and agar concentration. It seemed desirable, however, to investigate the significance of some other factors in this connection. In the present paper experiments are described where seedlings of different age and seedlings originating from seeds of different size were tested for their response to arginine. Furthermore, some earlier results concerning the effect of arginine on the nitrogen content of the seedlings, which seemed to need corroboration, were reinvestigated and extended.

Methods

The experiments were arranged in exactly the same way as earlier described (Fries 1954). The seedlings were always grown in culture tubes containing 10 ml.

nutrient medium with 1.5 per cent agar. When not otherwise indicated the cultures were incubated for 15 days in complete darkness at $+25^{\circ}\text{C}$.

The seeds were all from the 1953 harvest and produced seedlings somewhat larger than the average of earlier experiments. Except for one experiment (No. 1062) only peas of the strain, »Torsdag III», were used.

Experiments

A. *The importance of the seed size*

Kruyt (1954) has called attention to the importance of the original seed size for the subsequent development of the seedling in experiments with decotylised pea plants. The significance of this factor for the response of decotylised, etiolated pea seedlings to arginine and other metabolites had not been considered in the earlier published investigations. Therefore, an experiment was performed where seeds of two different weight classes, »small» and »large» seeds, were tested. As a pea is never absolutely spherical, the size of the seeds was expressed in terms of mg. fresh weight rather than in mm diameter. The fresh weight of each small seed was less than 200 mg., that of each large seed more than 250 mg. The former group deviated more from the normal size used as experimental material, than did the latter group, since the average weight normally amounted to 246 ± 2.7 mg. (mean value based on 50 peas from a batch selected for use in another experiment).

The seeds of both classes were sterilised in the ordinary way, soaked in sterile water and brought to germination on agar plates. When transferred to the test tubes the decotylised seedlings from the two seed classes differed slightly as to the length of the radicle and more pronouncedly as to their dry weight.

In the experimental series, the effect of 0.3 mM arginine and glycine, separately and combined, was tested. The results of the experiment are summarized in Table 1. As regards the average growth rate of the shoot, this was about the same in all series, the standard errors being too great to permit any conclusions from the mean values. The growth rate of the main root, on the other hand, was significantly increased by the added amino acids: moderately by glycine, strongly by arginine, and most strongly by the combined substances. The same could be said about the effect on the increase in dry weight.

The response of the small-seed plants to the added substances was almost exactly the same as that of the large-seed plants, if the growth rates are considered, except that perhaps the latter plants were somewhat more strongly influenced by glycine than the former. However, as regards the dry

Table 1. *The significance of the seed size for the response of the decotylised seedlings.*

When transferred to the culture tubes at the start of the experiment the seedlings from the small seeds had a shoot length of c. 3 mm, a root (+hypocotyl) length of 31.9 mm, and a dry weight of 6.7 mg., whereas the corresponding values for the seedlings from the large seeds were: c. 4 mm, 33.3 mm, and 8.5 mg., all average values obtained from 20 determinations. The small seeds weighed less than 200 mg., the large seeds over 250 mg. each, the mean values of the fresh weight being 162 mg. and 274 mg., respectively. Each experimental series comprised 8 cultures. The concentration of each substance added was 0.3 mM.

Additions	Seed weight in mg.	Length in mm			No. of lateral roots	Dry weight in mg.		
		Shoot	Main root	Lateral roots		Shoot	Root system	Whole plant
No addition ...	< 200	96 \pm 5	82.1 \pm 2.7	33.8	5.6	4.9	4.2	9.1
Arginine.....		110 \pm 4	122.9 \pm 6.7	46.7	6.9	6.2	4.7	10.9
Glycine		102 \pm 6	94.3 \pm 6.7	52.8	9.3	5.9	4.7	10.6
Arginine + glycine ...		112 \pm 6	130.4 \pm 4.4	57.9	7.5	7.0	4.8	11.8
No addition ...	> 250	104 \pm 4	82.4 \pm 2.0	50.1	6.9	7.4	4.9	12.3
Arginine.....		108 \pm 3	124.1 \pm 4.8	62.3	8.0	7.8	5.6	13.4
Glycine		103 \pm 5	101.0 \pm 3.8	76.9	11.0	7.2	5.7	12.9
Arginine + glycine ...		113 \pm 1	138.9 \pm 4.0	46.4	6.8	8.2	5.7	13.9

weight the plants from the small seeds gave lower values throughout than those from the large seeds. The former were also inferior as to the number and total length of lateral roots per plant.

It can thus be concluded that the size of the seed has a long-lasting influence upon the seedling developed from the seed even when the cotyledons are removed as early as 66 hours after the start of germination. The weight of root system and shoot are thus in all cases influenced, while the growth rate of the shoot and the main root seem to be unaffected. Except for this last-mentioned observation the results agree essentially with those of Kruyt (1954) obtained in a similar way. Since the seedlings responded in the same manner to the active substances tested irrespective of their origin from small or large seeds, the seed size factor does not seem to affect the results of experiments of this sort to any considerable degree. If peas of all sizes had been included in the starting material of the experiments, instead of a rather strictly limited fraction as usually has been the case, this would probably not have altered the results essentially, although the mean values, particularly as regards the dry weight, must have been less reliable.

B. *The age of the seedling at decotylisation*

During the development of the seedling there is a continuous flow of nutrients from the cotyledons to the young shoot and root. If the cotyledons

Table 2. *The significance of the age of the seedling at decotylisation for its response to arginine.*

»Age of the seedling» means the period from the start of germination to the transfer of the seedling into the culture tube immediately after decotylisation, thus comprising the soaking and swelling in distilled water (always 18 hours) and the following development on agar plates (24, 36, 48, or 60 hours). The concentration of l-arginine, when added, was 0.3 mM. The number of cultures per series was 6 in Expt. 1062, and 8 in Expt. 1188. In Expt. 1062 the seed material was »Torsdag II», in Expt. 1188 »Torsdag III».

Shoot length, root length (incl. hypocotyl), and total dry weight of the seedlings after decotylisation was in Expt. 1188: a) 42 hours old seedlings: c. 3 mm, 5.1 mm, and 3.3 mg., b) 54 hours old seedlings: c. 4 mm, 15.5 mm, and 4.7 mg., c) 66 hours old seedlings: c. 4 mm, 32.6 mm, and 7.1 mg., respectively, all figures being mean values based on 10 determinations.

Expt. No.	Age of seedling in hours	Additions	Length in mm			No. of lateral roots	Dry weight in mg.		
			Shoot	Main root	Lateral roots		Shoot	Root system	Whole plant
1062	54	Control	65 ± 3	76.3 ± 4.8	69.2	11.2	4.6	3.7	8.3
	54	Arginine	60 ± 3	93.3 ± 2.0	11.5	3.2	4.0	3.4	7.4
	66	Control	102 ± 2	87.8 ± 3.9	87.3	11.7	5.8	3.7	9.5
	66	Arginine	92 ± 4	107.5 ± 2.8	28.8	6.5	5.8	4.2	10.0
	78	Control	113 ± 3	110.0 ± 9.6	70.0	11.8	6.8	4.1	10.9
	78	Arginine	112 ± 5	115.3 ± 4.9	45.7	6.8	7.1	4.8	11.9
1188	42	Control	56 ± 4	75.0 ± 4.3	31.6	7.5	3.8	3.0	6.8
	42	Arginine	55 ± 3	95.4 ± 2.7	33.3	6.7	4.1	3.5	7.6
	54	Control	78 ± 5	81.4 ± 3.9	36.9	7.4	4.8	3.9	8.7
	54	Arginine	77 ± 2	96.8 ± 3.9	52.0	9.5	5.2	4.3	9.5
	66	Control	107 ± 4	99.4 ± 7.8	57.4	10.4	7.6	5.1	12.7
	66	Arginine	115 ± 2	131.6 ± 4.0	52.0	10.9	7.7	5.6	13.3

are removed early, less material has been transferred than if decotylisation is performed later. It seems reasonable to assume, therefore, that the response of the decotylised seedling to various substances may, to a certain degree, depend on the age of the seedling at decotylisation.

The importance of this factor was examined in two experiments, No. 1062 (part of which has already been published in Fries 1954, Table 1, p. 10) and No. 1188. In the former experiment the age of the seedling at decotylisation was 54, 66, or 78 hours, in the latter 42, 54, or 66 hours. In both cases the effect of arginine was tested.

Table 2 shows that the addition of arginine in all series caused an increase in the growth rate of the main root and usually also in the dry weight of the seedling. This increase, however, was very slight and not statistically significant in the oldest seedlings, which had been decotylised at an age of 78 hours. In all series with younger seedlings the effect on the root length was about equally strong, amounting to 20—30 per cent above the control.

Table 3. *The distribution of the soluble and the insoluble nitrogen fractions between shoot and root in decotylised seedlings grown with and without arginine.*

In addition to the quantity of nitrate-N contained in the nutrient medium each culture tube was supplemented with either a mixture of extra nitrates (1095 $\mu\text{g. Ca}(\text{NO}_3)_2 \cdot 4 \text{H}_2\text{O} + 274 \mu\text{g. KNO}_3$), or 3 micromoles (522 $\mu\text{g.}$) of L-arginine, the N-content thus being the same in all tubes.

At the end of the experiment the length of the shoot was 109 ± 3 mm in the nitrate series and 111 ± 4 mm in the arginine series, the length of the root 101.2 ± 4.5 mm and 130.3 ± 6.8 mm, respectively. Each series comprised 10 cultures.

N fraction tested	Addition	$\mu\text{g. N per plant}$			N content, % of dry wt.		
		Shoot	Root system	Whole plant	Shoot	Root system	Whole plant
Soluble N	Nitrate	253	51	304	3.24	0.98	2.34
	Arginine	325	75	400	4.09	1.37	2.97
Insoluble N	Nitrate	132	85	217	1.69	1.64	1.67
	Arginine	179	111	290	2.25	2.01	2.15
Total N	Nitrate	385	136	521	4.93	2.62	4.01
	Arginine	504	186	690	6.34	3.38	5.12

Thus, as far as arginine is concerned the age of the seedling when decotylised does not seem to influence the strength of the response, unless the age exceeds 66 hours. However, since only arginine has been tested, the possibility that the effect of other metabolites may be more dependent on this age factor has still to be considered.

C. *The effect of arginine on the nitrogen content of the seedlings*

If 3 micromoles (=522 $\mu\text{g.}$) of arginine are added to the culture medium the seedling contains considerably more nitrogen after 15 days than a control seedling in a medium without arginine (Fries 1954, Table 26). Three further experiments were performed in order to obtain information as to the form in which this extra nitrogen occurs in the arginine-fed seedlings. A distinction was made between soluble («non-protein») and insoluble («protein») nitrogen, trichloroacetic acid being used as the protein precipitating agent. The separation was made according to the method described by Keyssner and Tauböck (1933).

The earlier observed effect of arginine on the nitrogen content of the seedlings also appeared in these three further experiments. Although the amount of inorganic nitrogen in the basal medium obviously was very close to the optimum, the control cultures in two of the experiments were supplemented with an additional quantity of nitrate to make the nitrogen content equal to that of the test cultures with arginine added. The effect of arginine

Table 4. *Nitrogen content of seedlings derived from extremely small seeds and cultivated with and without arginine and glycine.*

The material for these analyses was obtained from the experiment recorded in Table 1 as »small seeds» with a fresh weight <200 mg.

N fraction tested	Days of incubation	Additions	µg. N per plant	N content, % of dry wt.
Soluble N	0	—	244	3.64
	15	No addition	231	2.54
		Arginine	333	3.06
		Glycine	278	2.62
		Arginine + glycine	358	3.03
Insoluble N	0	—	196	2.92
	15	No addition	171	1.88
		Arginine	214	1.96
		Glycine	209	1.97
		Arginine + glycine	233	1.97
Total N	0	—	440	6.56
	15	No addition	402	4.42
		Arginine	547	5.02
		Glycine	487	4.59
		Arginine + glycine	591	5.00

on both growth and nitrogen content manifested itself to about the same degree whether the control had received this supplementary quantity of nitrate or not.

In the first experiment where the soluble and insoluble nitrogen fractions of the seedlings were separated (Table 3), the shoot (minus hypocotyl) and the root system (plus hypocotyl) were kept apart in the analysis. In all cases a positive effect of arginine was noted. Not only did the arginine-fed seedlings show a larger absolute quantity of nitrogen per individual, but they also contained a higher percentage of both soluble and insoluble nitrogen. The shoot contained considerably more soluble nitrogen than insoluble, while in the root the situation was the opposite. Arginine increased both fractions to about the same degree.

In this first experiment the seeds used belonged to the normal weight class, c. 240–250 mg. In order to see if a divergent result was to be obtained when seedlings from smaller peas were analysed, the material presented in Table 1 was used. This was derived from peas lighter than 200 mg. Only entire seedlings were analysed (Table 4). In this experiment the effect of arginine manifested itself more strongly in the soluble than in the insoluble fraction, and was, on the whole, weaker than in the last-mentioned experiment. Glycine produced an increase chiefly in the insoluble nitrogen fraction. The

Table 5. *The distribution of the two nitrogen fractions between shoot and root of the decotylised seedlings after different periods of incubation with and without arginine.*

The material for analysis was five plants in each series. The additions of nitrate and l-arginine were the same as in Table 3. When transferred to the culture tubes at the start of the experiment the seedlings had a c. 4 mm long shoot, a 36.1 mm long root (incl. hypocotyl), and the dry weight was 7.5 mg. of each seedling.

N fraction tested	Days of incubation	Addition	µg. N per plant			N content, % of dry wt.		
			Shoot	Root system	Whole plant	Shoot	Root system	Whole plant
Soluble N	0	Control	—	—	262	—	—	3.49
	10	Nitrate	184	103	287	2.79	2.06	2.48
		Arginine	285	104	389	4.01	1.89	3.09
	15	Nitrate	292	73	365	3.79	1.33	2.77
		Arginine	312	101	413	3.80	1.58	2.83
	20	Nitrate	304	66	370	3.54	1.20	2.62
Arginine		309	73	382	3.51	1.26	2.62	
Insoluble N	0	Control	—	—	262	—	—	3.49
	10	Nitrate	73	101	174	1.11	2.02	1.50
		Arginine	114	102	216	1.61	1.85	1.71
	15	Nitrate	108	101	209	1.40	1.84	1.58
		Arginine	120	129	249	1.46	2.02	1.71
	20	Nitrate	122	113	235	1.42	2.06	1.67
Arginine		131	128	259	1.49	2.20	1.77	
Total N	0	Control	—	—	524	—	—	6.98
	10	Nitrate	257	204	461	3.90	4.08	3.98
		Arginine	399	206	605	5.62	3.74	4.80
	15	Nitrate	400	174	574	5.19	3.17	4.35
		Arginine	432	230	662	5.26	3.60	4.53
	20	Nitrate	426	179	605	4.96	3.26	4.29
Arginine		440	201	641	5.00	3.46	4.39	

effect of both substances together was almost exactly the same as of arginine alone.

The object of the third, and last, analytical experiment was to follow the changes in nitrogen content of the seedlings during a certain period of time. The effect of 3 micromoles per tube of arginine was compared with that of nitrate representing the same amount of nitrogen. Starting with 30 seedling cultures of either sort, 10 were analysed after 10 days, 10 after 15 days, and 10 after 20 days. The result is summarized in Table 5 and Figure 1.

By coincidence the seedlings originally contained exactly the same quantity soluble as insoluble nitrogen, viz. 3.5 per cent. The absolute amount of soluble nitrogen increased during the first ten days — slowly in the control seedlings and quickly in the seedlings with arginine. In both series about the same level of c. 380 µg. nitrogen per plant was attained after 20 days. The absolute amount of insoluble nitrogen, on the other hand, went down con-

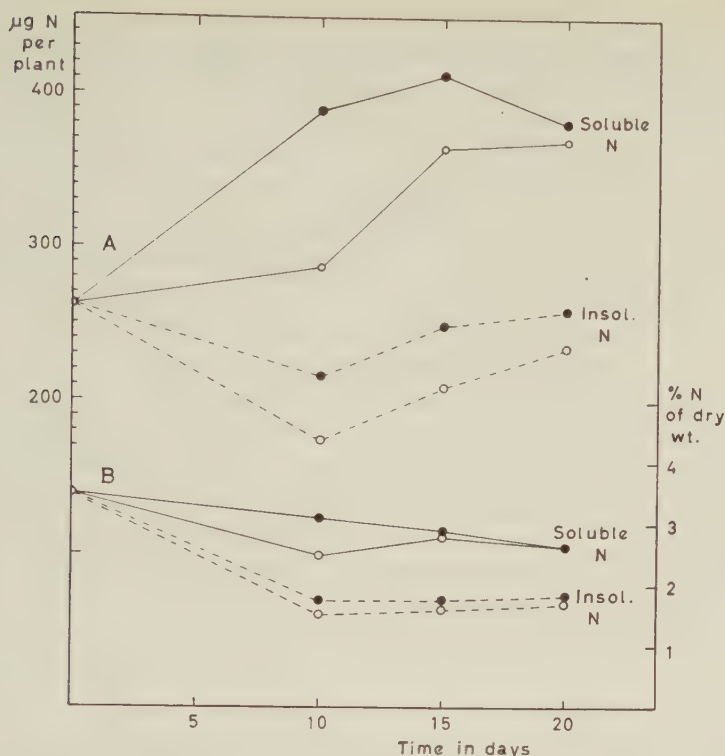


Figure 1. *Changes in nitrogen content of decotylised pea seedlings grown in darkness for 20 days with and without arginine. A: µg. N per plant; B: per cent N of dry weight. The open circles indicate plants grown with nitrate-N only, the filled circles plants to which part of the nitrate had been exchanged for l-arginine. Unbroken lines: soluble N; broken lines: insoluble N. Experimental conditions same as in Table 5.*

siderably during the first ten days, particularly in the control plants, but then rose slowly to about the original value. The control plants always contained less than the arginine-fed plants.

If the nitrogen content is expressed in terms of per cent of the dry weight the difference between the two experimental series becomes less striking. It appears then, that the control plants assumed a definite content of soluble nitrogen (c. 2.6 per cent) already after ten days, while the content of the arginine plants decreased more slowly from the original 3.5 per cent. As to the insoluble nitrogen, which comprises the protein fraction, there was a rapid decrease from 3.5 per cent to a final, fairly constant value of 1.6 to 1.8 per cent, the arginine plants always being a little above the control plants.

As regards the distribution of the two nitrogen fractions between shoot

and root the soluble nitrogen was by far more abundant in the shoot and the insoluble more abundant in the root, which agrees with the result of the above mentioned experiment recorded in Table 3.

Discussion

The experiment first reported in this paper demonstrates that decotylised seedlings responded to arginine and glycine in about the same way, irrespective of the size of the seed from which the seedling originated. Furthermore, the age of the seedling when decotylised did not affect the response to arginine, unless it exceeded 66 hours at the time of this operation. These results confirm the statement made in an earlier paper: »that the activity of arginine manifested itself when the conditions of the experiments were changed in one way or another» (Fries 1954, p. 28).

An analysis of the nitrogen content of the seedlings revealed certain interesting differences between the seedlings grown with nitrate as the only source of nitrogen and those grown in a medium where part of the nitrate (25 per cent, in terms of nitrogen) had been exchanged for arginine. If a distinction was made between nitrogen compounds soluble in trichloroacetic acid and those insoluble in this protein-precipitating solution the arginine-fed seedlings always contained more of both fractions than did the controls, although this difference was small in terms of per cent or $\mu\text{g. N per mg. dry weight}$.

As shown by a particular experiment the situation changed during the early development of the young pea plant. If the approximation is made that the nitrogenous substances insoluble in trichloroacetic acid are identical with proteins, the following picture can be drawn of the nitrogen economy of the decotylised seedling during the first 20 days of growth in the dark: The proteins present at decotylisation and probably just transferred from the cotyledons are rapidly broken down. The products, mainly amino-acids, are partly exuded into the medium and partly retained within the plant, particularly in the developing shoot. New proteins, however, are being synthesized all the time especially in the shoot and root tips, and after ten days an equilibrium is reached which maintains an almost constant protein level in the seedling.

The plants supplied with arginine exhibit a much higher content of soluble nitrogen than the control plants after the first ten days of cultivation. This surplus, which then gradually disappears, most probably consists of either absorbed arginine or non-protein compounds derived from arginine. It seems justified to assume that this fraction serves as a particularly suitable material

for protein synthesis in darkness, thereby also making possible a higher rate of growth. Such an interpretation of the growth-promoting effect of arginine would be based on the unproved assumption that protein synthesis is the process which in the first place limits the rate of growth in the dark under the experimental conditions. However, the hypothesis is supported by the fact that arginine does not influence the growth of decotylised seedlings cultivated in the light, where protein synthesis, at least in the shoot, is likely to proceed under more favourable conditions.

Summary

Decotylised seedlings of pea (*Pisum sativum* L.) were grown under aseptic conditions in a synthetic agar medium in the dark with the technique described earlier (Fries 1954). The main object of the experiments was to study the growth-promoting effect of arginine.

Seedlings developed from small seeds responded in the same way to arginine and glycine as did seedlings from large seeds. The age of the seedling at decotylisation did not affect the strength of the response to arginine, unless the seedlings were more than 66 hours old.

The nitrogen content was higher in those seedlings which had been cultivated with part of the nitrate exchanged for arginine (0.3 mM). The changes in the protein and non-protein nitrogen content were followed during the early development of the seedlings grown with and without arginine. The results are discussed.

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References

- Fries, N.: Limiting factors in the growth of the pea seedling root. — *Physiol. Plant.* 6: 292. 1953.
- Chemical factors controlling the growth of the decotylised pea seedling. — *Symb. Bot. Upsal.* 13, No. 1: 1. 1954.
- Keyssner, E. & Tauböck, K.: Die Stickstoffbilanz. Bestimmung der Stickstoff-Fractionen in der Pflanze. — *Handb. d. Pflanzenanalyse* herausgeg. v. G. Klein, Bd. 4, Teil 3: 1345. 1933.
- Kruijt, W.: A study in connection with the problem of hormonization of seeds. — *Proefschrift.* Amsterdam 1954.

Evaluation of the Growth Activity of Naphtalene Derivatives

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Problem

The discussion of the relation between chemical structure and growth activity of compounds of the auxin family has led to several theories, briefly reviewed by Hansen. These fit in well with the behaviour of most compounds, but there are noteworthy exceptions to all theories. One reason may be that what is called growth is not a physiologically uniform process. An attempt to study this problem has been made by Hansen, who has sorted out different morphologically separate growth reactions of roots. Another method, which has been employed on the one hand by McRae and Bonner, on another by Linser and Kaendl, is to treat the growth activity curves statistically. (With activity curve is implied in the following any curve showing the relation between the concentration of a compound and its growth effect.) They should be of a certain shape, and combinations of compounds should change this differently if the compounds have physiologically identical effects than if they have not.

In the present study an attempt has been made to follow up this line of approach for four closely related compounds, which partly form exceptions in Hansen's and others' experiments. They are *1-naphthaleneacetic acid*, *2-naphthaleneacetic acid*, *1-naphthoxyacetic acid*, and *2-naphthoxyacetic acid*.

The principle has been to study in a standard root growth test the action of all four compounds in all possible combinations over their whole range

of activity, and to try to interpret the activity curves obtained. — It should be noted that only the total root growth has been measured, and preliminarily no attempt has been made to distinguish between cell elongation proper and growth by cell multiplication. For technical reasons this was unfeasible in the study comprising about 800 tests run in quintuplicate.

Methods and evaluation of the results

The test method employed has been the root growth micro test developed by Lexander, giving the rate of root elongation after 24 hours of treatment in a complete nutrient solution and under strictly standardized conditions. The plant material was *Eroica* wheat.

The values given in figures 2 to 8 are computed in per cent of the growth without additions of growth compounds. In figure 2 the directly obtained values have been presented, in figures 3 to 8 they have been corrected in the following way. If root growth is inhibited by a non-toxic auxin it is decreased to a low level, which cannot be passed without killing the roots. There is a small residual growth independent of growth compounds and probably consisting only of cell divisions, swelling and the like, not equivalent to a real elongation. In order to eliminate this residual growth, in controls amounting to 3 mm. or about 25 per cent, a constant value of 3 mm. for 24 hours was subtracted from all figures. This does not change the shape of the curves but gives a better idea of the magnitude of the effects obtained. All points are averages of usually four independent tests combined at random. No attempt was made to smooth out the curves.

Neither of the two methods of interpreting the activity curves is unprejudiced, but both are founded on assumptions of a certain physico-chemical background.

McRae and Bonner have employed the principle of activators and inhibitors of enzyme reactions and assume that an activity curve can be presented as a kinetic curve according to Michaelis. The interaction between two compounds may appear as in figure 1 A, showing the interaction between an activator, X, and an inhibitor, Y, given in concentrations increasing from 1 to 3. The kinetic curves should converge toward a point on the ordinate; if the experimental results do so, it proves a competitive inhibition, i.e., that both compounds act at the same point. The shapes of the curves should be the same even if Y is an activator with the concentration increasing from 3 to 1. McRae and Bonner have also pointed out that the effect of Y must decrease with increasing [X]. — Figure 1 B shows an instance of a wholly additive effect of X and Y; each has a certain growth effect independent of the other. It is surprising to find that this can lead to the same type of kinetic curves as instance A. The reason is that curves converging toward the ordinate always are obtained if the difference between the growth effects of the two compounds is large, and growth approaches an upper limit; the curves will rise steeply in any scale but lie close together, and the inverted growth values will rapidly become small and seemingly constant, i.e., the kinetic curves converge rapidly. The activity curves may converge or run parallel, but the details are blurred by the inversion. The course of the kinetic curves will depend mainly on the position of a few growth values at low concentrations of X, and the further course of the curves

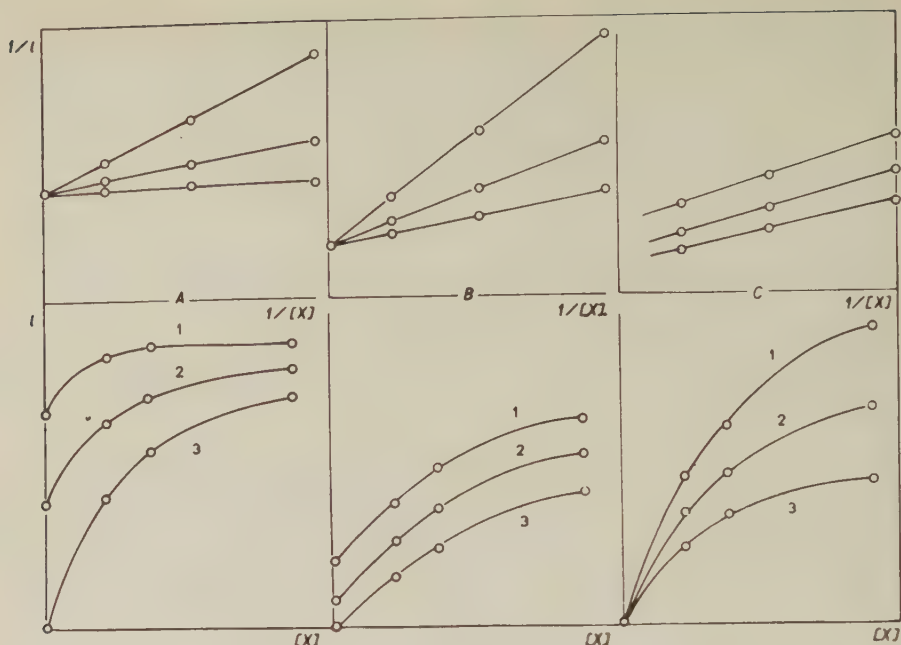


Figure 1. *A theoretical study of the interaction between two growth substances X and Y.* — *Lower graphs:* the relation between $[X]$ and rate of elongation (l) at three concentrations of Y ; *upper graphs:* the same material treated kinetically, showing the relation between $1/[X]$ and $1/l$. — *A:* X and Y are assumed to have physiologically identical effects, being able to substitute each other; if Y is an inhibitor the curves illustrate true competitive antagonism between the compounds. — *B:* X and Y act wholly independently of each other, the growth effects are additive. — *C:* the action of Y increases in the presence of X and vice versa. — The kinetic curves indicate for *A* and *B* »competitive action» (action in the same biochemical system), for *C* independent action.

is rather immaterial. This can also be inferred from the material of Housley et al., which seems to illustrate that even very irregular activity curves treated kinetically can be approximated into straight lines converging on the ordinate.

In McRae and Bonner's example with IAA and 4ClIB the course of the curves in figure 3 depends on the fact that the growth approaches an upper limit and on the position of the growth values for the lowest concentrations of IAA in table 3. These were for the extreme concentrations of 4ClIB

4ClIB	IAA	
	0.02	0.05
0	0.59	1.14
1.0	0.33	0.68
inhibition by 4ClIB 1.0	0.26	0.46

If, however, the activity curves diverge toward infinity (figure 1 C) more or less parallel kinetic curves are obtained, which according to McRae and Bonner indicate that the compounds do not act at one point. — The physiological interpretation of the curves in figure 1 C is, however, that if Y is an inhibitor, the inhibition increases with increasing [X], or, if Y is an activator, a synergism exists between the compounds. In neither case are the effects of the compounds independent of each other. Curves of this kind are obtained, for example, if the effect depends upon the ratio between the compounds, which clearly indicates an intimate interaction between them. The first instance can also be kinetically interpreted so that X forms the substrate for Y; inhibition by Y thus cannot be obtained if $[X]=0$, and the inhibition must increase to some degree with [X], not decrease as postulated by McRae and Bonner. This also holds true within the critical range of concentration in their experiment quoted on p. 176.

Even diverging activity curves may thus indicate a close physiologic interaction between the two compounds. The only reliable indication of a physiologically independent, additive action of two compounds seems to be that the activity curves in combination tests run parallel over a fairly wide range of concentrations.

The other interpretation of the activity curves has been made by Kaindl on material of Linser's. Kaindl's complete formula for the interaction between two growth factors reads

$$Z = A(1 - e^{-k_1 c_1 e^{-l_2 \sqrt{c_2}}}) - B(1 - e^{-k_1 c_1 e^{-l_2 \sqrt{c_2}}}) - C(1 - e^{-k_2 \sqrt{c_2} e^{-l_1 c_1}})$$

under the assumption that the interaction depends upon an adsorption to two different sites and the probabilities of hits for each compound. — In one experiment especially analyzed by Kaindl the first term denotes the growth promotion by IAA, the second term its growth inhibition at high concentration, and the third term an inhibition by eosin. The exponents $-l_2 \sqrt{c_2}$ and $-l_1 c_1$ denote the action of eosin on IAA and IAA on eosin, respectively. Within the main part of the activity range, e.g., at the centre with eosin and IAA = 10^{-2} per cent, term 2 equals zero, and $e^{-l_1 c_1}$ practically 1. This means that IAA exerts no inhibiting action, and IAA does not change the eosin effect but that the opposite holds true. The term $l_1 c_1$ gains in importance if c_1 is high, but then the agreement between found and computed values becomes less satisfactory. The whole interaction between the growth compounds is expressed by the exponent $-l_2 \sqrt{c_2}$ in term 1; for the rest the terms 1 and 3 illustrate an independent action of the compounds. Linser concluded from a visual judgement of the activity curves that IAA and eosin mainly acted independently of each other owing to the parallel course of the curves, but that eosin had some effect on the IAA action. The extensive deductions founded on adsorption isotherms and probabilities of hits, as a matter of fact, do not give any further information about the interaction between the compounds.

The activity curves obtained in the present results are of the types A, B, and C together with a modification of A. If two inhibitors have identical action they give a system of curves as in A but converging from high values on the ordinate. — It should be emphasized that the activity curves in figures 3 to 8 are drawn in a logarithmic scale, but those in figure 1 in a linear one.

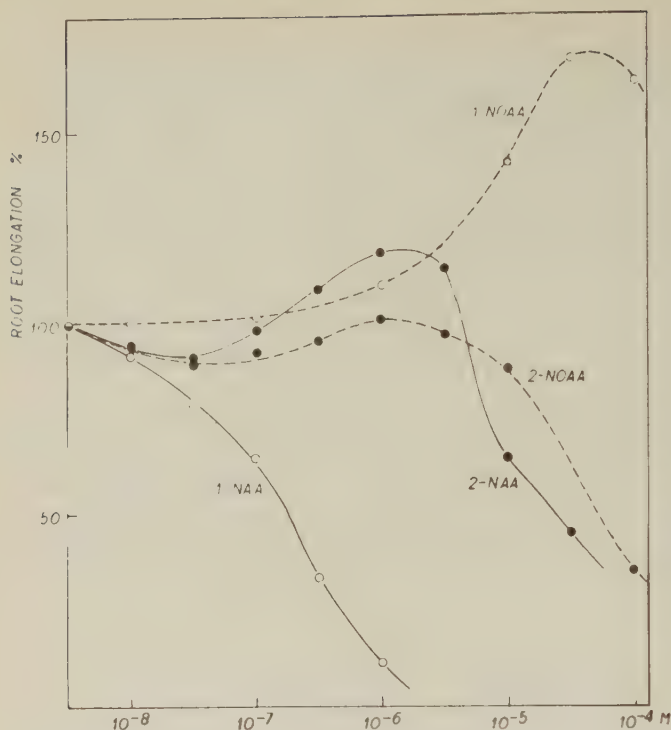


Figure 2. The root growth activity of the four investigated acids: 1-naphthaleneacetic acid (1-NAA), 2-naphthaleneacetic acid (2-NAA), 1-naphthoxyacetic acid (1-NOAA), and 2-naphthoxyacetic acid (2-NOAA). — Relative root elongation in 24 hours treatment, control=100.

Activity of the single compounds

All four substances have already been tested for their growth activity on roots and other materials (see Hansen, Åberg). The present results with the single compounds are presented in figure 2.

1-naphthaleneacetic acid has been unanimously characterized as an auxin and exerts in the present experiments only a growth-inhibiting action.

Little information is available about 2-naphthaleneacetic acid; it has been denoted as an auxin or an inactive compound, by Hansen as a weak auxin with root inhibitions above 10^{-6} M. This has been verified, but the activity curve has a more complicated course with a weak inhibition up to $3 \cdot 10^{-8}$ M, followed by a considerable growth increase, between $3 \cdot 10^{-8}$ and 10^{-6} M amounting to about 40 per cent.

1-naphthoxyacetic acid has been called inactive, auxin, and antiauxin; Åberg and Khalil found it to be inactive in itself but acting as an antagonist against external auxin, and Hansen has classified it as a root-auxin, with a strong growth promotion from 10^{-6} M. This has been verified and it can also be seen from figure 2 that the acid is wholly inactive below 10^{-6} M.

2-naphthoxyacetic acid has an activity curve resembling that of the 2-naphthaleneacetic acid; however, the increase in growth between 10^{-7} and 10^{-6} M is slight and growth never exceeds the control. It has been un-
animously called an auxin in the literature (see Hansen).

The following eight effects have thus been observed with the four acids:

1-naphthaleneacetic acid (1-NAA), growth inhibition only;

2-naphthaleneacetic acid (2-NAA), a weak inhibition (I) followed by a strong promotion (II) and a final inhibition (III);

1-naphthoxyacetic acid (1-NOAA), growth promotion only;

2-naphthoxyacetic acid (2-NOAA), a weak inhibition (I); an equally weak promotion (II), and finally, growth inhibition (III).

Interaction between the compounds

It has already been mentioned that the only way of elucidating whether two compounds have physiologically identical action or not is by means of a careful judgement of the activity curves, and that statistical methods fail or do not offer any advantages. The following principle has been assumed to be tentatively justified: (1) strictly parallel course of a series of activity curves indicates that the substances have physiologically different actions, (2) curves obviously converging at higher concentrations indicate identical action, and (3) diverging curves may show that some kind of interaction exists, it may be competition, synergism, or as an extreme case, that one action is totally subordinated another and disappears if the other one is present. Aberrant graph systems are obtained if one action wholly predominates (cf. figure 9).

It must further be kept in mind that both the maximum and the minimum of growth are limited by other factors than growth compounds. The nutrient conditions must determine an upper limit of growth, and the lower limit, of course, falls near zero.

(a) — The relation between 1-NAA and 1-NOAA (figure 3) ought to be fairly simple because each compound has only one action. The actions are not additive but the curves converge toward higher concentrations, which may imply some simple antagonism. The highest concentration of 1-NOAA (curve 5) falls outside the picture, however, probably for two reasons. According to figure 2 a growth inhibition sets in with this concentration, and Hansen has shown that it depends mainly upon a reduction in the cell multiplication. With this concentration of 1-NOAA and low values of 1-NAA the root growth also reaches its upper limit. This curve is thus sharply cut off on this level. A point which has not been investigated further is the indica-

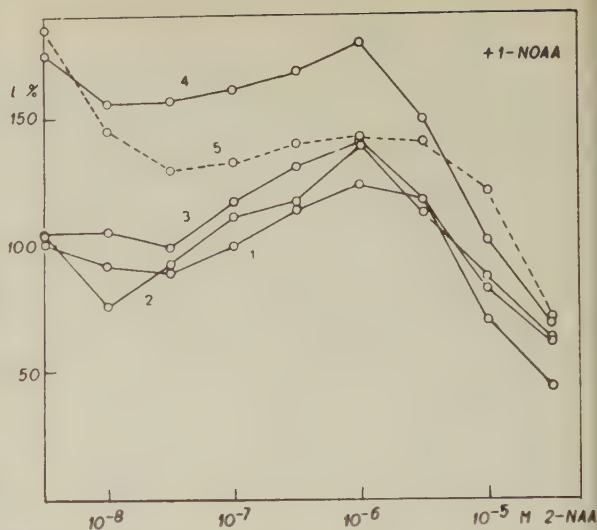
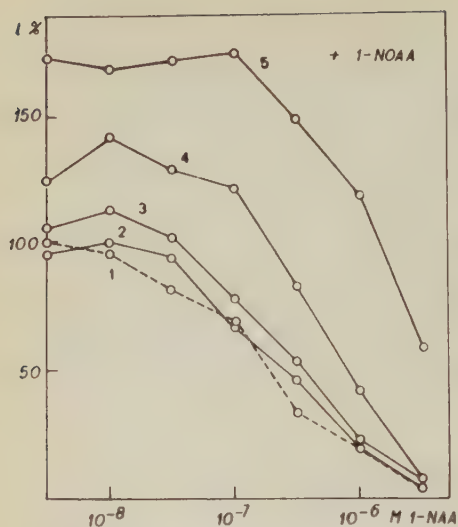


Figure 3. The interaction between 1-naphthaleneacetic acid (1-NAA) and 1-naphthoxyacetic acid (1-NOAA). — On the ordinate root elongation, control=100. Concentrations of 1-NOAA, curve 1=0, 2= 10^{-7} , 3= 10^{-6} , 4= 10^{-5} , 5= 10^{-4} M.

Figure 4. The interaction between 2-naphthaleneacetic acid (2-NAA) and 1-naphthoxyacetic acid (1-NOAA). — As figure 3. Concentrations of 1-NOAA, curve 1=0, 2= 10^{-7} , 3= 10^{-6} , 4= 10^{-5} , 5= 10^{-4} M.

tion of a peculiar synergism at low concentrations of both acids. — Nevertheless, the results show that a close interaction between the compounds occurs.

(b) — The interaction between 1-NAA and the inhibiting action (no. III) of 2-NOAA is still more obvious from figure 7. Above 10^{-6} M 2-NOAA the curves regularly converge, showing that the two inhibitors substitute each other and probably have the same kind of action.

(c) — Nevertheless the weak positive action (no. II) of 2-NOAA seems to remain unaltered even at the highest concentration of 1-NAA, indicating an additive effect of these two actions. No conclusions can be drawn regarding the weak first effect (no. I) of 2-NOAA.

The relation between 1-NAA and 2-NAA (figure 5) is important for the interpretation of all compounds. It is obvious that effect no. II of 2-NAA is flattened out and shifted toward higher concentrations on an addition of 1-NAA, which ought to occur if the combined action depends on, for example, the ratio between the compounds. Some close interaction occurs between these two activities. It is also interesting to note that action no. III of 2-NAA cuts off all curves along approximately the same line. The curves are partly

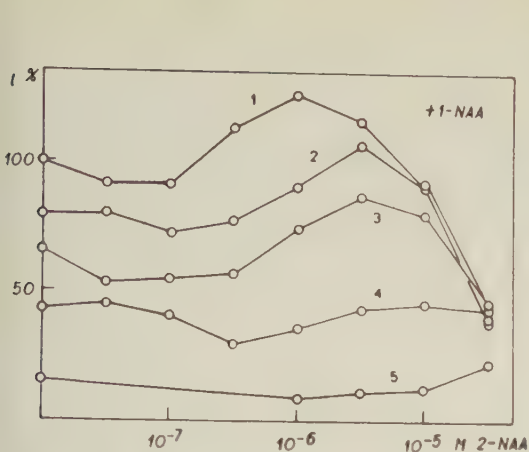


Figure 5. The interaction between 2-naphthaleneacetic acid (2-NAA) and 1-naphthaleneacetic acid (1-NAA). — As figure 3. Concentrations of 1-NAA, curve 1=0, 2= $3 \cdot 10^{-8}$, 3= 10^{-7} , 4= $3 \cdot 10^{-7}$, 5= 10^{-6} M.

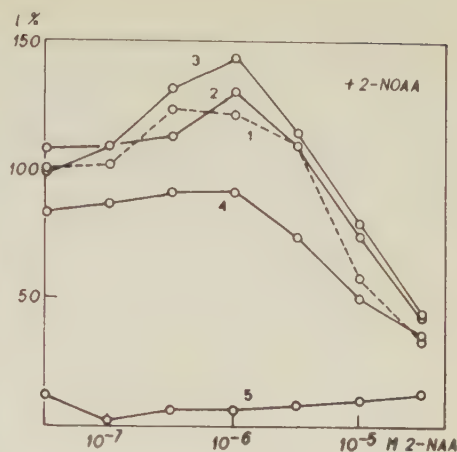


Figure 6. The interaction between 2-naphthaleneacetic acid (2-NAA) and 2-naphthoxyacetic acid (2-NOAA). — As figure 3. Concentrations of 2-NOAA, curve 1=0, 2= 10^{-7} , 3= 10^{-6} , 4= 10^{-5} , 5= 10^{-4} M.

very similar to those with IAA and eosin analyzed in detail by Linser and Kaindl. An attempt has therefore been made to explain them in the same way, as the result of one promoting and one inhibiting action of 2-NAA shifted toward the right in the presence of 1-NAA. The calculation has, however, been carried out graphically (figure 9) not numerically.

It has been assumed that curve 1 in figure 9 — copied from curve 1 in figure 5 — forms the difference between one showing growth promotion and another of growth inhibition. The former — curve 1 a — has been given a probable shape and position with a total increase of 75 per cent, which seems to be the upper limit in these experiments. The inhibition curve (1 b) has been obtained by subtracting 1 from 1 a. This has been repeated with curves 3 and 4 also taken from figure 5; the growth promotion curves 3 a and 4 a have been given exactly the same shape as 1 a and placed in reasonable positions. The computed inhibition curves 3 b and 4 b fall surprisingly near 1 b. With regard to the very strong influence 1-NAA must have upon the position of the curves of growth promotion and the roughness of the computations this justifies the conclusion that the growth inhibition by 2-NAA (action no. III) is independent of 1-NAA, and perhaps the latter is subordinated under the first-mentioned effect.

The graphical considerations thus indicate:

(d) — that the actions of 1-NAA and no. II of 2-NAA are physiologically closely related and competing, but that

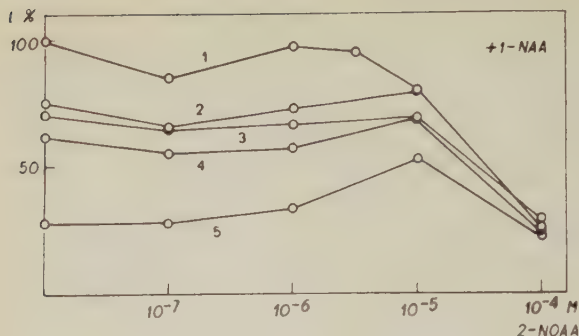


Figure 7. The interaction between 2-naphthoxyacetic acid (2-NOAA) and 1-naphthaleneacetic acid (1-NAA). — As figure 3. Concentrations of 1-NAA, curve 1=0, 2= 10^{-8} , 3= $3 \cdot 10^{-8}$, 4= 10^{-7} , 5= $3 \cdot 10^{-7}$ M.

(e) — 1-NAA and no. III of 2-NAA are independent of each other.

From the conclusions under these five points it ought to be possible to postulate the kinds of interactions in all other combinations of these compounds.

It should follow from (a) and (b) that 1-NOAA and 2-NOAA no. III would compete, which also appears satisfactorily from the converging curves above 10^{-6} M 2-NOAA in figure 8. The highest concentration of 1-NOAA again forms an exception. The same figure also shows clearly that the actions of 1-NOAA and nos. I and II of 2-NOAA are additive, which tallies with the conclusions under (a) and (c).

Figure 4 shows that the positive action, no. II, of 2-NAA is slowly inhibited by increasing 1-NOAA, but that action I would remain unaltered. The difference between figures 4 and 8 is interesting. Increasing 1-NOAA regularly inhibits the fairly strong positive action of 2-NAA but not the very weak action no. II of 2-NOAA. In agreement with the conclusions under (a) and (d) it can thus be assumed that action II of 2-NAA and that of 1-NOAA are physiologically similar. The highest concentration of 1-NOAA again behaves aberrantly. The course of the curves above 10^{-6} M in figure 4, on the contrary, seems to indicate an additive action, which tallies with the conclusions under (a) and (e) leading to the assumption that these two are physiologically independent.

There then remains the complicated interaction between 2-NAA and 2-NOAA each with three effects (figure 6). The interpretation is difficult because actions I and II of 2-NOAA are very weak and the inhibition no. III of 2-NAA very strong. The following conclusions can be tentatively drawn: 2-NOAA in concentration 3 exerts a fairly constant growth effect over the whole range of concentrations of 2-NAA; the effects are obviously additive. Action no. II of 2-NOAA is independent of all other effects studied. — The

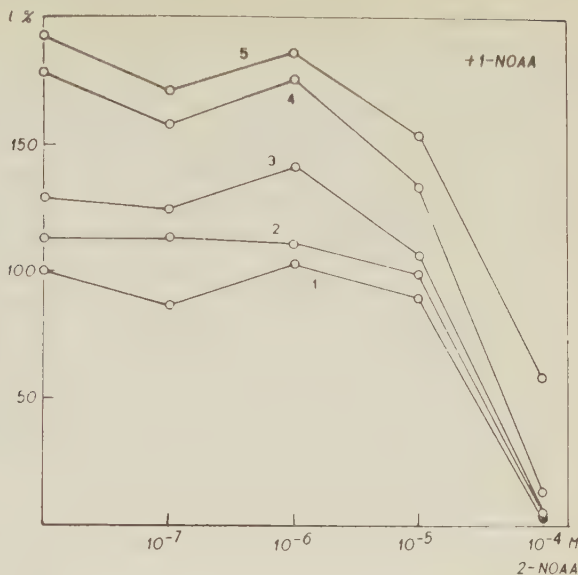


Figure 8. The interaction between 2-naphthoxyacetic acid (2-NOAA) and 1-naphthoxyacetic acid (1-NOAA). — As figure 3. Concentrations of 1-NOAA, curve 1=0, 2= 10^{-7} , 3= 10^{-6} , 4= 10^{-5} , 5= 10^{-4} M.

inhibiting action of 2-NOAA, no. III, cuts off sharply the entire positive effect of 2-NAA, it acts much more strongly in this respect than the similar activity of 1-NAA (figure 5).

There are otherwise some similarities between figures 5 and 6. The decreasing part of curve 4 of figure 6 has been plotted in figure 9 (as 6 b) together with the constructed hypothetical inhibition curves of 2-NAA. It fits in surprisingly well with the others with regard to slope and general position. This is interesting because if this concentration of 2-NOAA cuts out the entire actions I and II of 2-NAA, curve 4 in figure 6 must attain this shape provided that the inhibiting action of 2-NAA remains unaltered. The rather good agreement between curves b in figure 9 supports the fact that neither 1-NAA nor 2-NOAA can affect the inhibiting action of 2-NAA. According to the conclusions under (b) and (e) this should not be the case, either. It would seem that the inhibiting action of 2-NAA were of a very dominant nature and superimposed over other actions.

There thus seems to be a reasonably good agreement between the six series of experiments and they justify the following conclusions:

The final inhibiting action of 2-NAA is physiologically separated from the others, likewise the weak actions at low concentrations of 2-NAA and 2-NOAA; a complete mutual interaction of antagonism and synergism is exerted by the remaining four effects: the inhibiting actions of 1-NAA and 2-NOAA together with the positive effects of 2-NAA and 1-NOAA.

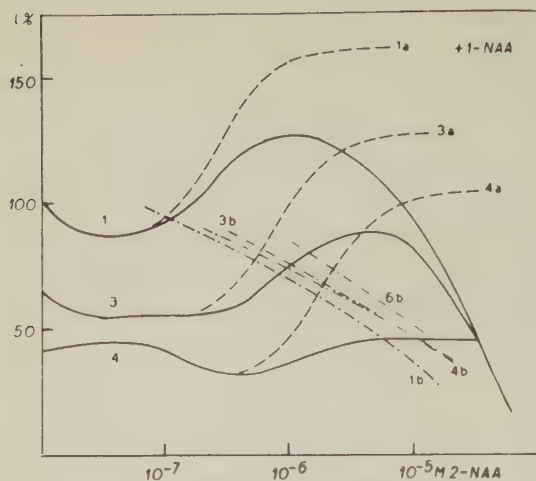


Figure 9. Graphical evaluation of the interaction between 1-NAA and 2-NAA (cf. figure 5 and the text). — Curves 1, 3, and 4 are identical with nos. 1, 3, and 4 respectively in figure 5; 1 a, 3 a, and 4 a are assumed curves of growth promotion; 1 b, 3 b, and 4 b computed curves of inhibition. Curve 6 b is identical with part of curve 4 in figure 6.

Discussion

The presented results have led to the conclusion that not all growth-increasing and growth-inhibiting effects of this group of closely related acids are physiologically comparable. Some show a mutual interaction which appears as an antagonism, in other instances the effects are additive. In the former case it has been assumed that the physiologic reactions are similar or identical, and it may even be so that the interaction has the character of a competitive inhibition in a biochemical sense (McRae and Bonner). In the author's opinion it is impossible, however, to prove that this is the case only from the shape of the activity curves or from graphical or numerical computations. On the other hand, it is likely that such a competition occurs, but it may be located to the absorption or transport of the substances as well as to the centre of activity. Audus has also doubted that conclusive proofs can be given of such a specific kind of interaction because the physiologic growth process is more complicated than a single biochemical reaction. It is more probable that such an antagonism does not occur if the activity curves are strictly additive. It should also be pointed out that the phenomenon described by McRae and Bonner as a competitive inhibition has been given another mathematical interpretation by Kaindl.

That competition does not occur only at the point of action of the compounds is clearly indicated by the fact that growth promotion by 2-naphthaleneacetic acid can be totally annihilated both by a high and in itself inhibiting concentration of 2-naphthoxyacetic acid and by a high but growth-promoting concentration of 1-naphthoxyacetic acid. The latter also counteracts both promoting and inhibiting actions of other acids. When it comes to

such less specific activities of compounds in relatively high concentrations it is probably necessary to consider competition or some other kind of hindrance during the absorption or transport of the compounds. Blackman and Robertson-Cuninghame have recently emphasized that the interaction between IAA and 2,4-D can neither be explained as wholly additive, nor as an ideal competition, even if such occurs, and Housley, Bentley and Bicks have stressed the permeability and transport factors in evaluating kinetic data.

This has a consequence for the classification and naming of these compounds. According to the definitions laid down by a committee (Tukey et al.) an antiauxin is a compound «which inhibits competitively the action of auxins». Now it is obvious from the foregoing discussion and has been emphasized already by Lineweaver and Burke that it is impossible to show definitely that such a competition takes place, not to mention where it takes place; it can perhaps only be shown that it does not occur in a given instance. For diagnostic reasons the term anti-auxin could strictly not be used at all, especially not for compounds supposed to antagonize the native auxin (Burström). Hansen has proposed another terminology. Substances in themselves inactive but counteracting only externally added auxins are called auxin antagonists, and it is inferred that their competition can hardly take place at the point of action, because they do not antagonize the native auxin. Substances in themselves active as root-growth promoters and also counteracting external auxins are called root auxins, owing to the similarity between their actions on roots and the action of auxins on shoots. This terminology seems to be most appropriate, since the too rigid definitions mentioned prohibit the use of the term anti-auxin.

It is generally assumed that 1-naphthaleneacetic acid, the synthetic auxin mostly resembling indoleacetic acid, exerts an action identical with that of the natural auxin. Judging from the found interactions this might be true also of 2-naphthoxyacetic acid in its growth-inhibiting action, and the growth-promoting effects of 2-naphthaleneacetic acid and 1-naphthoxyacetic acid should be antagonistic; this assumption seems to be likely because the two last-mentioned acids as root auxins probably antagonize also the native auxin. Street has compared 1-naphthaleneacetic acid, 1-naphthoxyacetic acid, and 2-naphthoxyacetic acid on excised tomato roots and concluded that only the last-mentioned acid resembles indoleacetic acid. The 1-substituted acids act as mutual antagonists, as in our experiments, but do not show interaction with indoleacetic acid. This would complicate the picture further.

On the other hand, the root-growth inhibition by 2-naphthaleneacetic acid should be physiologically of another kind. It is also striking that its activity curve is steep, the inhibition sets in rapidly, and it has the power of effectively cutting off other growth effects. It seems to be a growth inhibition attacking some vital point in the growth mechanism, but not the same one as IAA and its equivalents.

The fallacy of the mentioned definitions seems to be clear again when it appears that there is no simple term for the natural cell elongation hormones (IAA and its associates). »Growth hormone» includes growth of every kind and »auxin» synthetic compounds, which sometimes must be kept apart from the natural ones.

Of still another nature are the weak effects of low concentrations of 2-naphthoxyacetic and 2-naphthaleneacetic acids. Shifts from positive to negative growth effects or vice versa with increasing concentrations have sometimes been observed, even if apparently toxic effects are excluded. They have given rise to speculations about weak auxins giving anti-auxin effects in high concentrations or, as formulated by Åberg and Khalil, »residual auxin effects» of anti-auxins. A certain precaution seems to be necessary in interpreting such phenomena since it appears that not all effects are of physiologically similar nature. It is hardly justified to base elaborate theories on the mere course of activity curves without a critical examination of the nature of the effects observed. It should only be mentioned that if the auxins are fixed both to activity centers as assumed by McRae and Bonner and others, and to inactivating systems, as suggested by Brian and Rideal, and an antagonist competes at both sites, this could lead to rather complicated modes of actions and shifts between activities without any auxillary hypotheses. This is the leading principle of Linser and Kaindl, and the complex actions of the 2-substituted derivatives might conform to such a picture. Further speculations are premature, however.

With regard to the problem of the relation between structure and activity of the compounds, these should be classified according to the actions related to that of the natural auxin, or supposed to be so. Other effects are immaterial in this connexion. Thus 1-naphthaleneacetic acid and 2-naphthoxyacetic acid should be called auxins, and 2-naphthaleneacetic acid and 1-naphthoxyacetic acid active as root auxins or auxin antagonists. The differences between the naphthalene and naphthoxy acids have already been pointed out by Veldstra. He has given an explanation of the higher auxin activity of 1-naphthalene- and 2-naphthoxyacetic acids founded on the sterical behaviour of the side chain, in accordance with his general view of this problem. This would account for a lower activity of the other two compounds but hardly for a strong antagonistic effect, since the steric differences, especially between the naphthoxy acids, seems to be small. Åberg has compared the four acids on flax roots, which are more susceptible to auxins and hardly react significantly against growth-promoting compounds. He has for that reason observed only growth inhibitions by all four acids, missed the other effects, and consequently founded the explanations on an incomplete material.

Two points should be emphasized which may be pertinent to the structure-activity problem:

(1) — Both naphthaleneacetic acids are strongly active between 10^{-7} and 10^{-6} M, both naphthoxyacetic acids equally active between $3 \cdot 10^{-6}$ and $3 \cdot 10^{-5}$ M. *The quantitative effect depends only upon the type of linkage between ring and side chain.*

(2) — A displacement of the side chain causes a complete reversal of the activity from a positive to a negative one within each pair of acids. *The qualitative response depends also upon the location of the side chain.* The shift in activity resembles a physiologic all-or-none reaction more than something depending upon a quantitative competition for places in an active system.

Summary

(1) — A study has been made of the root growth activity of 1-naphthaleneacetic, 2-naphthaleneacetic, 1-naphthoxyacetic, and 2-naphthoxyacetic acids, and with all reciprocal combinations of the acids.

(2) — The following effects were observed: 1-NAA, only growth inhibition; 2-NAA, with increasing concentration a weak inhibition, a rather strong growth promotion, and a final growth inhibition; 1-NOAA, growth promotion only; 2-NOAA, a weak inhibition followed by a weak promotion and a strong inhibition.

(3) — It has been concluded that the actions of 1-NAA, 1-NOAA, the final inhibition by 2-NOAA and the promotion by 2-NAA are physiologically identical or mutually antagonistic.

(4) — The final inhibition by 2-NAA and the weak effects at low concentrations of 2-NAA and 2-NOAA are of a different nature.

(5) — Thus 1-NAA and 2-NOAA should be classified as auxins, 2-NAA and 1-NOAA as equally strong antagonists or root auxins.

(6) — The position of the side chain only changes the qualitative not the quantitative effect of the acids.

(7) — Some implications for the interpretation of growth activity curves in general and for the structure-activity problem have been pointed out.

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References

- Åberg, B.: On optically active plant growth regulators. — Kungl. Lantbrukshögskol. Ann. 20: 241. 1954.
— & Khalil, A.: Some notes on the effect of auxin antagonists and synergists upon coleoptile growth. — Id. 20: 81. 1953.

- Audus, L. J.: Auxin antagonists and synergists. A critical approach. — *New Phytol.* 53: 461. 1954.
- Blackman, G. E. & Robertson-Cuninghame, R. G.: Interactions in the physiological effects of growth substances on plant development. — *J. exper. Bot.* 5: 184. 1954.
- Brian, R. C. & Rideal, E. K.: On the action of plant growth regulators. — *Bioch. Biophys. Acta* 9: 1. 1952.
- Burström, H.: Studies on growth and metabolism of roots. VI. — *Physiol. Plant.* 4: 470. 1951.
- Hansen, B. A. M.: A physiological classification of »shoot auxins» and »root auxins» I—II. — *Botan. Not.* p. 230. 1954.
- Housley, S., Bentley, J. A. & Bickle, A. S.: Studies on plant growth hormones III. — *J. exper. Bot.* 5: 373. 1954.
- Kaindl, K.: Zur Wirkungsweise von Wuchs- und Hemmstoffen II. — *Biochem. Biophys. Acta* 6: 395. 1951.
- Lexander, K.: Growth-regulating substances in the roots of wheat. — *Physiol. Plant.* 6: 406. 1953.
- Lineweaver, H. & Burk, D.: The determination of enzyme dissociation constants. — *J. Amer. Chem. Soc.* 56: 658. 1934.
- Linser, H.: Zur Wirkungsweise von Wuchs- und Hemmstoffen. I. — *Bioch. Biophys. Acta* 6: 384. 1951.
- McRae, D. H. & Bonner, J.: Chemical structure and antiauxin activity. — *Physiol. Plant.* 6: 485. 1953.
- Street, H. E.: Factors controlling meristematic activity in excised roots VI. Effects of various 'antiauxins' on the growth and survival of *Lycopersicum esculentum*, Mill. — *Physiol. Plant.* 8: 48. 1955.
- Tukey, H. B., Went, F. W., Muir, R. M. & van Overbeek, J.: Nomenclature of chemical plant regulators. — *Plant Physiol.* 29: 307. 1954.
- Veldstra, H.: Researches on plant growth substances IV. — *Enzymologia* 11: 97. 1944.

The Role of Ketoglutarate and Polyphenol Oxidase in the Synthesis of Melanin during Morphogenesis in *Blastocladiella emersonii*

By

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Introduction

In recent years, intensive investigations of the aquatic Phycomycete, *Blastocladiella emersonii*, have led to some insight into the biochemical basis for morphogenesis in this organism (Cantino, 4, 6, 7, and references therein). In brief, one morphogenetic pathway (that leading to the formation of thick-walled, brown, resistant sporangia) is initiated and maintained by bicarbonate which apparently interferes with decarboxylation in the tricarboxylic acid cycle and thus brings about the accumulation of increased concentrations of alpha-ketoglutarate within the organism. Concomitantly, various shunt mechanisms are initiated which lead to the synthesis of new materials associated with the composition and architecture of the plant; one such substance is a melanin-like pigment localized in the thick, chitinous wall of the resistant sporangium. It is the purpose of this report (1), to partially elucidate the general nature of the coupling between the imposition of the bicarbonate »trigger mechanism« and the initiation of the shunt mechanisms which ultimately lead to melanin synthesis, and more specifically (2), to present direct evidence that the ketoglutarate which accumulates within the plant at the onset of morphogenesis does, indeed, play a direct and causal role in the initiation and maintenance of reactions responsible for melanin synthesis during morphogenesis.

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Materials and Methods

Cultures of *Blastocladiella emersonii* (Cantino and Hyatt, 10) were grown in liquid medium PYG^{1/2}B (Cantino, 4), whose composition was as follows: peptone (Difco), 1.25 g., yeast extract (Difco), 1.25 g., glucose, 3.0 g., NaHCO₃, 1.0 g., *aq* brom cresol purple (0.4 per cent), 0.8 ml., and distilled water to make 1000.0 ml. For this particular investigation, it was necessary to obtain large quantities of resistant-sporangial plants in liquid culture. After some manipulation of environmental conditions, it was established that this could be achieved by growing the fungus between ca 25° and 30° C. either (1), in 150 ml. of medium PYG^{1/2}B in 250 ml. Erlenmeyer flasks on a rotary-tilting type Ross-Kershaw shaker (180 oscillations per minute) or (2), in 4000 ml. of medium PYG^{1/2}B in 6000 ml. flasks fitted with an aeration device through which a continuous stream of air was bubbled vigorously during growth. Aeration was sufficiently rapid to maintain all fungus thalli thoroughly suspended and constantly agitated. Under these conditions, the usually-flocculent and dispersed type of growth is not obtained; instead, there are formed discrete, compact, dark-brown, roughly-spherical, hollow colonies which consist of hundreds of resistant-sporangial plants tightly entangled with one another by rhizoids (Figure 1). When a normally-flocculent, dispersed type of growth of thin-walled plants was required, cultures were incubated in essentially the same manner except that the basal medium did not contain bicarbonate.

Cultures were harvested by suction on filter paper in Büchner funnels and washed thoroughly with distilled water (ca 2000 ml./4.0 gm. wet weight); for preparation of dry powders, the washed fungus mats were dessicated under vacuum over calcium chloride at 5° C. for 24 hours, ground to a uniform, brown powder in pre-chilled mortar and pestle, and refrigerated at 0° C. over calcium chloride in closed tubes. Such dried powders, as well as freshly-harvested mats of whole plants, were homogenized as needed in order to prepare enzymatically-active, cell-free extracts. Although ordinary glass homogenizers were perfectly satisfactory for the preparation of extracts from thin-walled plants (Cantino and Hyatt, 8), they could not be used with suspensions of resistant-sporangial plants: the thickness and stickiness of the latter rendered homogenization tedious and slow, and furthermore, the thick-walled sporangia were seldom broken in the process. Therefore, in the earlier experiments, the plant material was homogenized for ca 15 minutes in pre-chilled mortar and pestle with acid-washed sand or powdered glass (about two parts dry weight of powder or whole cells to one part of sand or glass in 25 ml. of 1.4×10^{-1} M phosphate, pH 7.0). Cells were also disrupted with a sonic oscillator (Raytheon) in one experiment. Finally, we evolved a simple technique whereby powders and whole plants (0.4 g. dry weight of powder or its equivalent of whole plants/10.0 ml. buffer) were homogenized for one or two hours in a modified "ball mill" bathed in an ice bath and powered by a magnetic stirring device (Figure 2). A grinding mixture made up of 10 per cent carborundum (finest grade) and 90 per cent powdered glass facilitated the complete disintegration of the resistant sporangia. One part of grinding mixture was used per two parts of dried powder or its equivalent of whole plants.

Homogenates were then dialyzed (with exceptions as noted in the context) against running tap water (ca 25° C.) for six to eight hours, or against 1.4×10^{-1} M phosphate buffer, pH 7.0, at 4° C. for twenty to twenty-four hours, with one change of buffer. Dialyzed homogenates were then centrifuged (with exceptions as noted in



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Figure 1. Spherical colonies of resistant-sporangial plants of *B. emersonii*, derived from 10-day old shake-cultures grown on medium $PYG^{1/2}B$.

Figure 2. Modified ball mill used for homogenization of resistant sporangial plants of *B. emersonii*. A teflon-coated bar magnet, placed upon glass beads in the 125 ml. flask and powered by a magnetic stirring device from below, was rotated approximately 500 to 900 times/minute.

the context) at $500\times G$ and $1^{\circ} C$. for five minutes; supernatants derived therefrom were used directly for studies of oxidative enzymes. Homogenates and supernatants were always prepared immediately before use.

Acetone powders were prepared by mixing dialyzed or undialyzed supernatants with 10 volumes of acetone, centrifuging, and drying the white powder in a vacuum dessicator at $4^{\circ} C$.

Manometric studies were made with a conventional Barcroft-Warburg manometer (ca 120 oscillations/minute) using 2.0 ml. of reaction mixture and 0.2 ml. of 10 per cent KOH in the center well equipped with a filter paper wick. Contents of side arms were tipped in after ten minutes equilibration at $29^{\circ} C$.

Spectrophotometric studies were made with a conventional model DU Beckman spectrophotometer, using quartz cuvettes of 1 cm. light path. In certain experiments, a Klett-Summerson colorimeter was used.

For analytical purposes, reaction mixtures were deproteinized with zinc hydroxide; cleared supernatants were extracted continuously with peroxide-free ether, using a modified version (manufactured by Scientific Glass Apparatus Co., Bloomfield, N.J.) of the apparatus described by Schmall *et al* (25). Control runs were made with organic acids of low partition coefficient (e.g. citric, *cis*-aconitic, isocitric, ketoglutaric, etc.) to determine the time required for a satisfactory recovery.

Chromatographic separation of organic acids in supernatants and ether extracts was accomplished by the method of Lugg and Overell (19), using a slightly-modified solvent system (*n*-butanol/aq. $4.0\times 10^{-1} M$ formic acid, equilibrated 48 hours before use), and 0.04 per cent alcoholic bromphenol blue, pH 6.8 for spraying. As a result, *rf* values differed slightly from those of Lugg and Overell as follows: (first value,

Lugg and Overell; second value, ours, averaged from 2 to 8 determinations) fumarate, 0.84/0.89; succinate, 0.73/0.74; lactate, 0.72/0.67; pyruvate, 0.70/0.66; oxalacetate, 0.65/0.64; ketoglutarate, 0.57/0.58; isocitrate, —/0.50; malate, 0.49/0.45; citrate, 0.41/0.37; *cis*-aconitate, —/0.40.

Characterizations and quantitative determinations of organic acids were made by fractional distillations, *rf* values, direct titrations, differential color reactions (Buch *et al*, 2), and the methods of Koepsell and Sharpe (17) and Miller and Muntz (22) as modified by Koenemann (16).

The standard, highly-purified, tyrosinase preparation (No. C 318; 10,000 catecholase units/ml.) was generously supplied by Dr. C. R. Dawson, Columbia University, New York. The refrigerated enzyme was diluted as needed to yield solutions containing either 100 or 200 catecholase units/ml. The hyaluronidase (850 T.R.U./mg.) was kindly supplied by Dr. Joseph Seifter, Wyeth Institute of Applied Biochemistry, Philadelphia.

Dihydroxyphenylalanine (dopa) was obtained from Nutritional Biochemicals Corp.; 2.0×10^{-2} *M* solutions, pH 7.0, were always prepared just before use. Tyrosine was obtained from Nutritional Biochemicals Corp. and made up as a 1.7×10^{-2} *M* solution in 1.7×10^{-2} *M* NaOH. Catechol was obtained from Merck and Co.; 9×10^{-3} *M* and 2.7×10^{-1} *M* solutions, pH 7.0, were freshly prepared for each experiment. The sources of all other chemicals were the same as those itemized in Cantino and Hyatt (8).

Experimental

Manometric studies. — Preliminary experiments established that whole-cell suspensions prepared from freshly-harvested resistant-sporangial plants oxidized catechol (in a catechol-hydroquinone system such as that used, for example, by Goddard and Holden, 14) at a reasonably rapid rate (Figure 6). At the end of an hour, final reaction mixtures were always red. In the absence of hydroquinone, rates for catechol oxidation were initially high but tapered off rapidly; in the absence of catechol, hydroquinone was not oxidized. Suspensions of dried powders derived from such plants displayed no significant loss of activity (Figure 6), thus greatly facilitating the preparation and subsequent storage of enzymatically-active material. Rates of oxygen consumption were roughly linear and directly related to the quantity of suspension used (Figure 3). With the relatively mild grinding techniques (mortar and pestle *without* added abrasives) used in the preparation of these suspensions, whether derived from powders or freshly-harvested plants, the rhizoid-bearing, basal cells were completely destroyed but virtually all resistant sporangia borne thereon remained unbroken. Thus, the results referred to above really represent rates of activity for suspensions of *intact* resistant sporangia.

Presumably, permeability barriers had been destroyed in the thoroughly dessicated but intact resistant sporangia in dried powders. And yet, when

suspensions were centrifuged (500 and 1500 \times G), supernatants derived therefrom possessed virtually no activity; over 90 per cent of the catecholoxidizing capacity was always recovered in the centrifugate. Identical results were obtained with suspensions incubated at 5° C. for as long as five hours before centrifugation, as well as with centrifugates which were resuspended and then re-centrifuged successively three or four times. These results suggested that the enzymes responsible for oxidation of catechol were more or less localized within, and firmly bound to, the walls of the resistant sporangia.

Subsequently, attempts were made to solubilize the enzyme by relatively mild procedures which might dissolve or partially disrupt the thick, chitinous walls of the resistant sporangia. For example, because of the presumed similarity between the building blocks of chitin and those of hyaluronic acid, suspensions of resistant sporangia were incubated for different time intervals, at 5 and 25 °C., in several concentrations of hyaluronidase. No dissolution of the walls was detected microscopically, and no increase in catechol-oxidizing activity was obtained in the supernatants derived from the suspensions of resistant sporangia treated in this fashion.

Finally, therefore, suspensions of resistant sporangia were treated more vigorously by grinding them for different time intervals with mortar and pestle, but in the presence of powdered glass. Homogenates were then centrifuged, and both supernatants and centrifugates were tested manometrically once again for their capacity to oxidize catechol. It was clear (Figure 5) that the virtually-complete disintegration of resistant sporangia following three minutes of grinding was directly related to a sudden, very great (ca 50-fold) increase in *total* (uncorrected for endogenous) activity of the supernatant and a relatively small (ca $\frac{1}{3}$ -fold) decrease in *total* activity of the centrifugate. When corrected for endogenous respiration in the absence of catechol, however, the results (not shown in figure) revealed that once again, most (ca 60 to 80 per cent) of the catechol-oxidizing activity had been retained in the centrifugate and that only ca 20 to 40 per cent had been recovered in the supernatant. Comparable results were obtained with suspensions, subjected to sonic vibration for ca one hour, wherein sporangia were completely disintegrated into very small wall fragments. Re-suspension of centrifugates, followed by further incubation at 4 °C. for various time intervals and re-centrifugation did not induce any further leaching out of activity; the supernatants derived from them were always devoid of catechol-oxidizing enzymes. It was concluded that the thick walls of the dehydrated and presumably-dead but intact resistant sporangia did impose a partial barrier upon the »dissolution» of the enzyme complex, but that nevertheless, even when such walls were thoroughly shattered, over 60 per cent of the enzyme

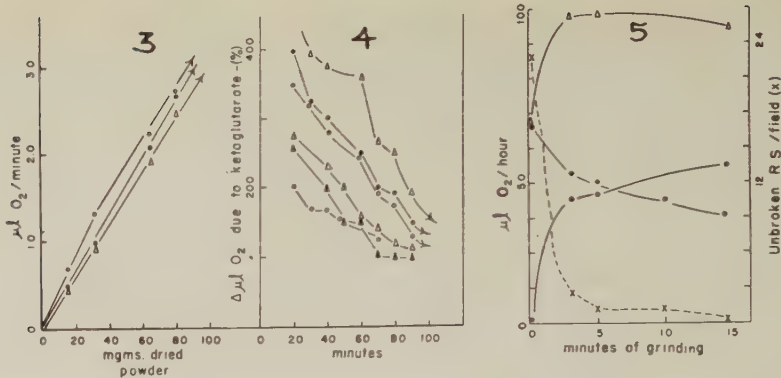


Figure 3. The rate of oxidation of catechol by different concentrations of suspensions of dried powders derived from resistant-sporangial plants. Each vessel contained 0.1 ml. each of 9×10^{-3} M catechol and 2.7×10^{-1} M hydroquinone (side arm), 0.6 ml. water, and 0.2, 0.4, 0.8, or 1.0 ml. suspension (0.42 g. powder/5.25 ml. 1.4×10^{-1} M phosphate, pH 7.0) plus sufficient 1.4×10^{-1} M phosphate to make 2.0 ml. total volume. Rates of oxygen consumption per minute were based upon average values of oxygen consumption after 10 minutes (top curve), 30 minutes (middle curve), and 60 minutes (bottom curve). Results are not corrected for endogenous respiration, in the absence of substrate, which averaged 15 to 20 per cent of rates delineated in the figure.

Figure 4. The time course of the effect of added ketoglutarate upon the oxidation of catechol by dialyzed, whole homogenates of dried powders derived from resistant-sporangial plants. Each vessel contained 0.1 ml. 9×10^{-3} M catechol, 0.1 ml. 2.7×10^{-1} M hydroquinone, and 0.2 ml. 10^{-1} M ketoglutarate (all 3 in side arm), 1.0 ml. homogenate (0.4 g. powder/10 ml. 1.4×10^{-1} M phosphate, pH 7.0), and water to make 2.0 ml. All results are corrected for endogenous respiration in the absence of catechol, with and without ketoglutarate. Data were selected from results of many experiments in order to illustrate the range of variation for the effect of ketoglutarate.

Figure 5. The effect of the length of homogenization-time upon the respiratory activity of dried powders derived from resistant-sporangial plants. Suspensions (0.4 g. powder/10.0 ml. 1.4×10^{-1} M phosphate, pH 7.0) were incubated at 4° C. for 60 minutes, and then homogenized in the presence of powdered glass with mortar and pestle for 3, 5, 10, and 15 minutes. The homogenates were examined microscopically for the presence of unbroken resistant sporangia (counts made on ca 15 fields, selected at random, at a magnification of ca 100 diameters) and then centrifuged for 5 minutes at $500 \times G$ at 1° C. Centrifugates were resuspended in 10 ml. of phosphate. Each vessel contained 0.1 ml. each of 9×10^{-3} M catechol and 2.7×10^{-1} M hydroquinone (side arm), 1.0 ml. of either the supernatant or the resuspended centrifugate, and water to make 2.0 ml. Results are not corrected for endogenous respiration in the absence of substrate. Open circles: supernatants. Shaded circles: resuspended centrifugates. Triangles: total activity, calculated from data for supernatants and centrifugates. Crosses: average number of unbroken resistant sporangia per field.

remained firmly bound to the chitinous, melanin-impregnated, fragments of these walls.

For comparative purposes, manometric measurements were made of the rate at which catechol was oxidized by highly-purified tyrosinase (containing 130 of Dawson's catecholase units; cf. Miller *et al*, 21 and Materials and Methods) in our conventional catechol-hydroquinone reaction mixtures. This concentration of enzyme was chosen because, in our hands, the rate of oxygen consumption based upon a 10-minute reaction time was more or less directly proportional to the enzyme concentration at levels of 10 to 130 catecholase units per vessel. Beyond this point, further increases in enzyme concentration progressively yielded disproportionately smaller and smaller increases in oxygen consumption. Under these conditions, then, it was determined that one unit of catecholase consumed ca 11 μ l. O_2 /hour (corrected for endogenous). On this basis, and assuming for the moment that oxidation of catechol by cell-free homogenates was due to the presence therein of a polyphenol oxidase with catecholase activity, numerous measurements revealed that our suspensions of dead, intact resistant sporangia generally contained from 150 to 180 catecholase units per gm. dry weight of plant material.

On the other hand, if our concepts (e.g. Cantino, 4, 5, 6, 7) about morphogenesis in *Blastocladiella* were essentially correct, then it followed that the colorless, thin-walled plants of the fungus should display little if any polyphenol oxidase activity. Numerous manometric measurements were therefore made with homogenates of freshly-harvested plants, as well as dried powders, derived from 4 to 12 day-old cultures of thin-walled plants. A variety of preparations was tested, including dialyzed and undialyzed acetone powders and dialyzed and undialyzed supernatants and whole homogenates prepared with and without the use of sand, glass, and carborundum. In every instance, the catecholase activity of such preparations was practically negligible (whether or not ketoglutarate was added; see next section) . . . from zero to 8, with an average of 4 units per gm. dry weight of plant material. The possibility that lack of polyphenol oxidase activity was due to the presence of some kind of an inhibitor was tentatively ruled out by the observation that homogenates of thin-walled plants did not inhibit the activity of cell-free preparations of resistant-sporangial plants. Thus, homogenates of thin-walled plants of *B. emersonii* did not possess the capacity to oxidize catechol. These results provided substantial, direct, and corroborative evidence for our notions that the appearance of the enzyme systems required for the synthesis of melanin is associated solely with the alternate developmental pathway, induced by bicarbonate, which culminates in the formation of brown, thick-walled, resistant sporangia.

Following these initial experiments, all subsequent homogenizations were performed with the apparatus illustrated in Figure 2; the procedure, involving a minimum of effort, insured the preparation of consistently very active homogenates in which all resistant sporangia were completely destroyed. It was soon discovered that the capacity of the homogenates to oxidize catechol (as well as tyrosine and »dopa») was completely lost following dialysis (lower curve, Figure 7). The addition of ketoglutarate to the reaction mixtures brought about an initial, almost-complete, recovery of the rates of activity originally found in the undialysed homogenates (Figure 7) (ketoglutaric acid, detectable in resistant sporangial plants (Cantino, 6), was presumably removed from the homogenates by dialysis). In the absence of catechol, alpha-ketoglutarate was not oxidized. Although the oxidation of catechol was inhibited by phenylthiourea (10^{-2} to $10^{-3} M$), rates and total quantities of oxygen consumed were not affected by (1), 10^{-4} to $4 \times 10^{-4} M$ KCN (a level quite effective in causing complete inhibition of the cytochrome oxidase activity in homogenates of thin-walled plants of this fungus (Cantino, 5; Cantino and Hyatt, 8); (2), $3 \times 10^{-2} M$ arsenite (when corrected for non-enzymatic reactions between arsenite and hydroquinone); (3), $3 \times 10^{-1} M$ malonate; and (4), 10^{-4} to $10^{-5} M$ DPN (diphosphopyridine nucleotide). The effect of ketoglutarate appeared to be rather specific in that other acids (pyruvic, citric, iso-citric, *cis*-aconitic, etc.), as well as vitamins (pyridoxal phosphate, biotin, thiamine, etc.) could not substitute for it, either individually or in various combinations. The magnitude of the stimulation brought about by ketoglutarate varied somewhat within relatively narrow limits, but its stimulatory effect was demonstrated in every one of numerous replicate experiments; representative results are delineated in Fig. 4.

Spectrophotometric studies. — Having established manometrically, *via* oxygen consumption, that ketoglutarate played some sort of a vital role in the enzymatic oxidation of catechol by homogenates of resistant sporangial plants of *Blastocladiella emersonii*, attempts were made to corroborate and to further elucidate the phenomenon spectrophotometrically. For this purpose, it was obviously impossible to employ whole homogenates. However, the foregoing manometric studies had established that supernatants derived from homogenates always possessed a small but significant amount of catechol-oxidizing activity; therefore, homogenates of dried powders were centrifuged at $500 \times G$, and the resulting supernatants were dialyzed against phosphate buffer and then used directly for the following spectrophotometric assays.

(*Experiments with cytochrome C*). — The enzymatic oxidation of catechol was conveniently followed by measuring the simultaneous reduction of cytochrome C at 550 m μ . (Actually, as explained below, reduction of cytochrome

C is non-enzymatic; it is dependent upon the enzymatic oxidation of catechol only because the latter generates the quinones with which the cytochrome reacts). In the absence of supernatant, or alternatively, in the absence of catechol, no reduction of cytochrome occurred (Figure 12). Once again, incorporation of ketoglutarate in the reaction mixtures not only increased the initial rates of reduction of cytochrome C, but it also maintained them at a reasonably constant value whereas without it, rates had tended to taper off (Figure 12). In the absence of catechol, ketoglutarate did not effect reduction of the cytochrome; in the absence of supernatant, no reaction occurred between ketoglutarate and catechol. As in the foregoing manometric experiments, the effect appeared to be specific in that other acids did not bring about the stimulation of activity. In fact, the addition of isocitrate to reaction mixtures containing ketoglutarate actually *depressed* the rates by about 30 per cent (these observations on the effect of isocitrate are not without significance; see discussion). Neither cyanide (10^{-4} M) nor DPN (10^{-4} M) had any effect upon the rates of reduction of cytochrome C when they were incorporated in reaction mixtures, irrespective of the presence or absence of ketoglutarate. In all of the foregoing experiments, the enzyme (supernatant) concentration was not limiting (Figures 16, 17).

(*Experiments with pyridine nucleotides*). — The catechol-oxidizing activity of cell-free preparations was also followed at 340 m μ *via* the enzymatic reduction of TPN to which the oxidation of catechol was coupled. In the absence of either catechol or supernatant, or alternatively, when the supernatant was boiled, no reduction occurred (Figure 8). DPN, on the other hand, was completely ineffective as an electron acceptor during oxidation of catechol. Although 10^{-5} M 2,4 dinitrophenol yielded no detectable inhibition, the reaction (as measured by the reduction of TPN) was inhibited 100 per cent by 10^{-2} M phenylthiourea. Once again, incorporation of ketoglutarate in the reaction mixtures consistently brought about a small but significant stimulation in the rate of reduction of TPN (Fig. 8) and, as with the cytochrome C experiments, the presence of the acid tended to prevent the rates from tapering off. Most significant, however, were the additional observations that the stimulatory effect of ketoglutarate was greatly accentuated when bicarbonate was also present in the reaction mixture (Figure 18).

(*Experiments with enzymatically-produced quinones*). — In view of the complexity of the presumed mechanism for the tyrosinase-catalyzed oxidation of catechol (Dawson and Tarpley, 13; cf. also Figure 19), the foregoing experiments could not provide definitive information about the actual site of action of ketoglutarate, nor the loci at which cytochrome C and TPN were reduced, in the reaction chain leading from catechol to its oxidation products. In the following experiments, therefore, we relied heavily upon

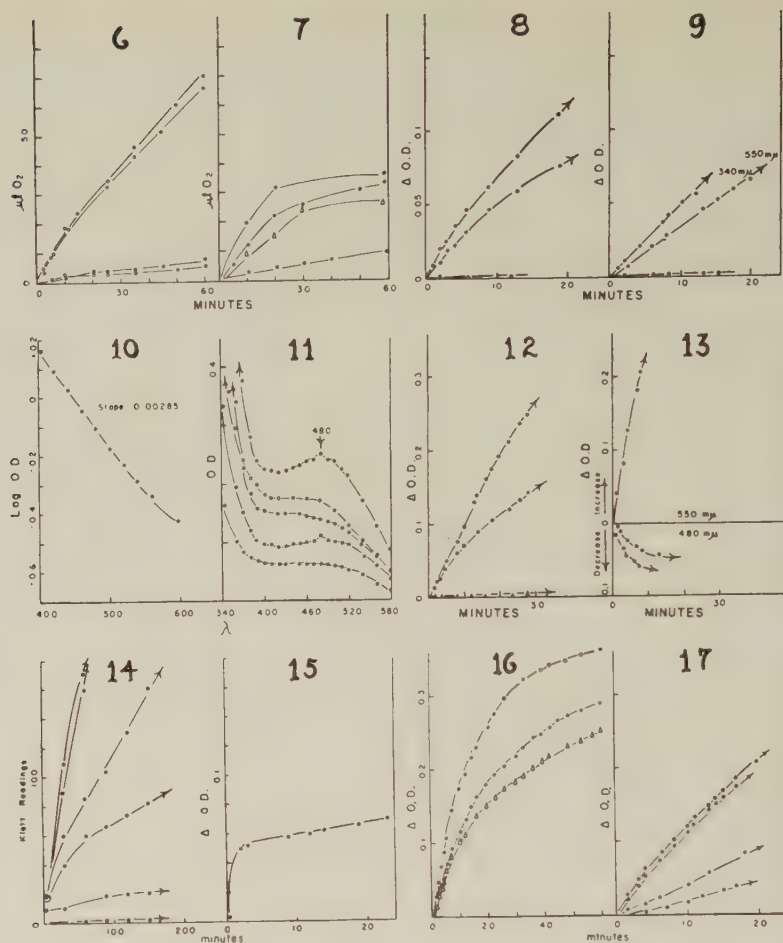


Figure 6. A comparison of the oxidation of catechol by suspensions of freshly-harvested resistant-sporangial plants with that by suspensions of dried powders derived from resistant-sporangial plants. Suspensions of freshly-harvested plants (4.07 g. wet weight/15.2 ml. 1.4×10^{-1} M phosphate, pH 7.0, equivalent to 0.4 g. dry weight/10 ml. phosphate) and dried powders (0.4 g./10 ml. phosphate) were prepared with mortar and pestle. Each vessel contained 0.1 ml. each of 9×10^{-3} M catechol and 2.7×10^{-1} M hydroquinone (side arm), 1.0 ml. of suspension, and water to make 2.0 ml. Two top curves: upper curve, freshly-harvested plants; lower curve, dried powders. Two bottom curves: endogenous respiration in the absence of substrate.

Figure 7. The effect of added ketoglutarate upon the oxidation of catechol, tyrosine, and dihydroxyphenylalanine by dialyzed homogenates of dried powders derived from resistant sporangial plants. Each vessel contained either 0.1 ml. each of 9×10^{-3} M catechol and 2.7×10^{-1} M hydroquinone, 0.2 ml. of 8.3×10^{-3} M tyrosine in 1.7×10^{-3} M NaOH, or 0.2 ml. of 2.7×10^{-2} M dihydroxyphenylalanine in the side arm, along with 0.2 ml. of 10^{-1} M ketoglutarate, and 1.0 ml. of homogenate and water in the main well to make

2.0 ml. Three upper curves: open circles, catechol and hydroquinone; shaded circles, tyrosine; triangles, dihydroxyphenylalanine. Bottom curve: maximum level of respiration obtained in the absence of ketoglutarate for all substrates.

Figure 8. *The effect of ketoglutarate upon the oxidation of catechol by dialyzed supernatants derived from homogenates (0.4 g./10 ml. phosphate) of dried, powdered resistant-sporangial plants as measured by the coupled reduction of TPN at 340 m μ . Each cuvette contained: 1.0 ml. 1.4×10^{-1} M phosphate, pH 7.0, 0.3 ml. supernatant, 0.3 ml. 2.7×10^{-1} M catechol, 0.25 ml. 2.5×10^{-3} M TPN, additions as noted below, and water to make 3.0 ml. Upper curves: open circles, 0.5 ml. 10^{-1} M ketoglutarate incorporated in reaction mixture; shaded circles, no additions. Lower curve: endogenous activity in the absence of catechol.*

Figure 9. *The oxidation of isocitrate by dialyzed supernatants, as measured by the coupled reduction of TPN at 340 m μ , and by the coupled reduction of cytochrome C, via TPN, at 550 m μ . Supernatants were prepared as for Figure 8. Each cuvette contained 0.5 ml. of 10^{-1} M isocitrate, 0.3 ml. supernatant, 1.0 ml. of 1.4×10^{-1} M phosphate, addition as noted below, and water to make 3.0 ml. Upper curves: shaded circles, 0.25 ml. 2.5×10^{-3} M TPN; open circles, 0.05 ml. 2.5×10^{-3} M TPN and 0.5 ml. 1.7×10^{-4} M cytochrome C. Lower curve: endogenous level in the absence of substrate.*

Figure 10. *The absorption spectrum of an alkaline solution of the pigment in dried, powdered resistant sporangia of B. emersonii.*

Figure 11. *The absorption spectra of the product of the enzymatic oxidation of catechol by dialyzed supernatants. Shaded circles (two lower curves): reaction mixtures contained 0.3 ml. supernatant, 0.3 ml. 2.7×10^{-1} M catechol, 1.0 ml. 1.4×10^{-1} M phosphate, pH 7.0, and water to make 3.0 ml; reaction mixtures were incubated 28 minutes (lower curve) and 137 minutes (upper curve) at 30° C. Shaded circles (two upper curves): reaction mixtures as outlined above except that they also contained 0.5 ml. 10^{-1} M ketoglutarate. Reaction mixtures were incubated 28 minutes (lower curve) and 137 minutes (upper curve) at 30° C. Open circles: reaction mixture contained 5.0 ml. 1.4×10^{-1} M phosphate, pH 7.0, 1.0 ml. 2.7×10^{-1} M catechol, and 0.05 ml. of tyrosinase (100 units of catecholase/ml.). Reaction stopped after 4 minutes.*

Figure 12. *The effect of ketoglutarate on the oxidation of catechol by dialyzed supernatants, as measured by the coupled, non-enzymatic reduction of cytochrome C at 550 m μ . Supernatants were prepared as for Figure 8. Each cuvette contained 0.3 ml. of supernatant, 1.0 ml. 1.4×10^{-1} M phosphate, pH 7.0, 0.5 ml. 1.7×10^{-4} M cytochrome C, 0.3 ml. 2.7×10^{-1} M catechol, additions as indicated below, and water to make 3.0 ml. Open circles: reaction mixture contained 0.5 ml. 10^{-1} M ketoglutarate. Shaded circles: no additions. Triangles: reaction mixture did not contain supernatant.*

Figure 13. *The reaction between cytochrome C and the oxidation products of catechol, as measured at 550 and 480 m μ . Supernatants were prepared as for Figure 8. Shaded circles: reaction mixtures containing 0.5 ml. supernatant, 1.7 ml. 1.4×10^{-1} M phosphate, 0.5 ml. 2.7×10^{-1} M catechol, and 2.3 ml. water were incubated 2 hours at 30° C. Aliquots (2.5 ml.) of above solution was then mixed with 0.5 ml. 1.7×10^{-4} M cytochrome C and read intermittently at 480 and 550 m μ . Open circles: reaction mixtures as above, but containing 0.5 ml. 10^{-1} M ketoglutarate.*

Figure 14. *The effect of aeration on the oxidation of catechol by dialyzed supernatants and by tyrosinase, as measured by color formation in the reaction mixtures. Supernatants were prepared as for Figure 8. All reaction mixtures contained 0.5 ml. 2.7×10^{-1} M catechol, 1.67 ml. 1.4×10^{-1} M phosphate, additions as indicated below, and water to make 5.0 ml.*

Two upper curves: both reaction mixtures contained 0.5 ml. tyrosinase (100 units catecholase/ml.) but one (shaded circles) also contained 0.84 ml. 10^{-1} M ketoglutarate while the other (open circles) did not. Four lower curves: all four reaction mixtures contained 0.5 ml. supernatant, and 0.84 ml. 10^{-1} M ketoglutarate, but one (upper curve) was aerated vigorously, a second (middle curve) not aerated, and other two (lower curves) made anaerobic in Thunberg tubes.

Figure 15. *The oxidation of reduced cytochrome C by ketoglutarate in the presence of dialyzed supernatants, as measured at 550 m μ .* Supernatants were prepared as for Figure 8. Cuvettes contained 0.5 ml. 1.7×10^{-4} M cytochrome C (reduced immediately before use with dithionate), 1.0 ml. 1.4×10^{-1} M phosphate, 0.5 ml. 10^{-1} M ketoglutarate, 0.2 ml. water, and 0.5 ml. phosphate-bicarbonate buffer, pH 6.8 (2.0 g. NaHCO_3 per 100 ml. 1.7×10^{-1} M phosphate, pH 5.7).

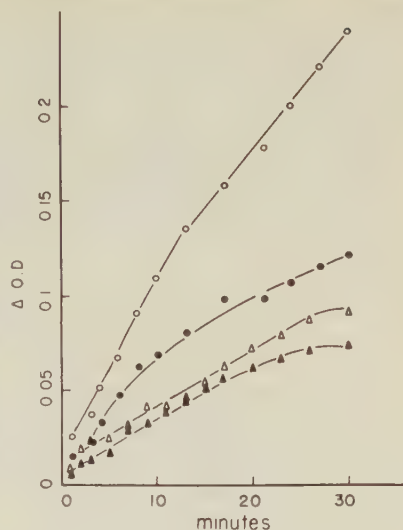
Figure 16. *The effect of different concentrations of catechol upon the enzymatic oxidation of catechol by dialyzed supernatants, as measured by the coupled, non-enzymatic reduction of cytochrome C at 550 m μ .* Each cuvette contained 1.0 ml. 1.4×10^{-1} M phosphate, 0.5 ml. 1.7×10^{-4} M cytochrome C, 0.3 ml. supernatant, 2.7×10^{-1} M catechol as indicated below, and water to make 3.0 ml. Shaded circles: 0.3 ml. catechol. Open circles: 0.1 ml. catechol. Triangles: 0.05 ml. catechol.

Figure 17. *The effect of different concentrations of supernatant upon the enzymatic oxidation of catechol, as measured by the coupled, non-enzymatic reduction of cytochrome C at 550 m μ .* Each cuvette contained 1.0 ml. 1.4×10^{-1} M phosphate, 0.5 ml. 1.7×10^{-4} M cytochrome C, 0.3 ml. 2.7×10^{-1} M catechol, supernatant as indicated below, and water to make 3.0 ml. Two upper curves: 0.5 ml. (open circles) and 0.3 ml. (shaded circles) of supernatant. Two lower curves: 0.1 ml. (open circles) and 0.05 ml. (shaded circles) of supernatant.

the observations that the absorption spectrum of o-quinone exhibits a peak at 390 m μ and then tapers off rapidly until at 480 m μ very little absorption occurs, whereas the spectrum for hydroxy-o-quinone has a well-defined peak in the vicinity of 480 m μ (cf. Tarpley, 28).

When supernatants were incubated aerobically with catechol, reddish oxidation products were formed; in the presence of ketoglutarate in otherwise identical reaction mixtures, far greater quantities were produced (Figure 14). Absorption spectra were then run on the aerobic reaction mixtures, both with and without the addition of ketoglutarate, after incubation for 28 and 137 minutes, respectively (Figure 11). The comparative optical densities revealed once again that ketoglutarate induced increased production of colored products. In addition, the spectra obtained after 137 minutes were characterized by a well-defined, highly reproducible peak at 480 m μ ; a peak, therefore, which apparently appeared only after the reaction was well under way. Spectra of reaction mixtures in which catechol had been oxidized by purified tyrosinase preparations never exhibited such a peak (Figure 11. and cf. Tarpley, 28 who was also unable to detect any piling

Figure 18. *The effect of bicarbonate upon the oxidation of catechol by dialyzed supernatants, as measured by the coupled reduction of TPN at 340 m μ . Supernatants were prepared as for Figure 8. Each cuvette contained: 1.0 ml. 1.4×10^{-1} M phosphate, pH 7.0, 0.3 ml. supernatant, 0.3 ml. 2.7×10^{-1} M catechol, 0.25 ml. 2.5×10^{-3} M TPN, additions as noted below, and water to make 3.0 ml. Shaded triangles: as above. Shaded circles: plus 0.5 ml. 10^{-1} M ketoglutarate. Open triangles: plus 0.5 ml. phosphate-bicarbonate buffer, pH 6.8 (2.0 g. NaHCO_3 per 100 ml. 1.7×10^{-1} M phosphate, pH 5.7). Open circles: plus 0.5 ml. phosphate-bicarbonate buffer and 0.5 ml. 10^{-1} M ketoglutarate.*



up of hydroxy-o-quinone in similar reaction mixtures). It was assumed that the 480 μ peak was due to the accumulation of hydroxy-o-quinone resulting from the enzymatic oxidation of catechol by cell-free preparations of *B. emersonii*.

Once again, incorporation of KCN had no detectable effect upon either reaction as measured colorimetrically, while phenylthiourea induced complete inhibition. Oxygen was apparently essential for the production of these chromogens; vigorous aeration increased the rates of their production whereas in the absence of oxygen, virtually no color developed (Figure 14). Furthermore, without oxygen, neither ketoglutarate nor carrier quantities of TPN had any effect upon the course of the reaction as judged by these criteria. When colored catechol-quinone mixtures were prepared enzymatically as described above, boiled, and then mixed with ketoglutarate, no change occurred in their absorption spectra; apparently, ketoglutarate did not react non-enzymatically with either catechol or colored quinones. It was clearly established, however, that oxygen could be replaced by TPN as the electron acceptor in the primary oxidation of catechol (Table 1). In fact, under anaerobic conditions, the quantity of TPN reduced at the expense of catechol oxidation is approximately twice that reduced under aerobic conditions.

Next, short time spectrophotometric experiments revealed that when aerobic reaction mixtures containing enzyme and catechol were followed at 390 $m\mu$, no appreciable change in optical density could be detected. When TPN was incorporated along with the other ingredients, a small but significant rise in optical density (ca. 0.05) occurred, after which the readings

Table 1. *The reduction of TPN via the oxidation of catechol under aerobic and anaerobic conditions at 30° C.* Each vessel contained 0.25 ml. of 2.5×10^{-3} M TPN, 1.0 ml. of 1.4×10^{-1} M phosphate buffer, pH 7.0, 0.3 ml. of supernatant, and water to make 3.0 ml. In addition, vessels 2 and 3 contained 0.36 ml. of 2.7×10^{-1} M catechol. Vessel 3, a Thunberg tube, was evacuated prior to tipping in the supernatant from the side arm.

Minutes of incubation	Change in optical density at 340 m μ		
	Vessel No. 1 (control)	Vessel No. 2 (aerobic)	Vessel No. 3 (anaerobic)
30	0.013	0.084	0.158
60	0.012	0.098	0.179

at 390 m μ leveled off to a constant value. But, when ketoglutarate and TPN were added simultaneously, then once again there was no detectable change at 390 m μ . In both of the two latter experiments, optical density levels at 340 m μ (reduced TPN peak) rose continuously at rates similar to those delineated in Figure 8. We interpreted the results to mean that during the early stages of the oxidation of catechol and the concomitant reduction of TPN, small, transient quantities of o-quinone had tended to pile up momentarily; in the presence of ketoglutarate, however, the accumulation of the o-quinone was prevented.

When the enzymatically generated catechol-quinone reaction mixtures whose absorption spectra showed a well-defined peak at 480 m μ were mixed with cytochrome C, the latter was rapidly reduced once again (Figure 13); in fact, cytochrome C was reduced far more rapidly than it had been when it was incorporated in fresh reaction mixtures containing only catechol and no accumulated, colored oxidation products (e.g., cf. rates shown in Figure 12). Simultaneously, judging from the rapid decrease in optical density at 480 m μ , the hydroxy-o-quinone rapidly disappeared (Figure 13). But, when ketoglutarate was incorporated into these mixtures along with cytochrome, the rate of decrease in optical density at 480 m μ was repressed by about 50 per cent. In the absence of added cytochrome C, the enzyme-catechol-quinone system yielded no appreciable change at 480 m μ unless ketoglutarate was also present, in which case a slight increase (ca 0.03 to 0.04) in optical density could always be detected at 480 m μ . Finally, results identical with those shown in Figure 13 were also obtained when the pre-formed catechol-quinone reaction mixtures were boiled prior to the addition of cytochrome C. Thus, whereas it was clear that catechol and cytochrome did not react directly with one another non-enzymatically, it was equally certain that cytochrome did react non-enzymatically with one or more of the colored quinones which, in turn, had been derived *via* the enzymatic oxidation of catechol by cell-free extracts of *Blastocladiella*.

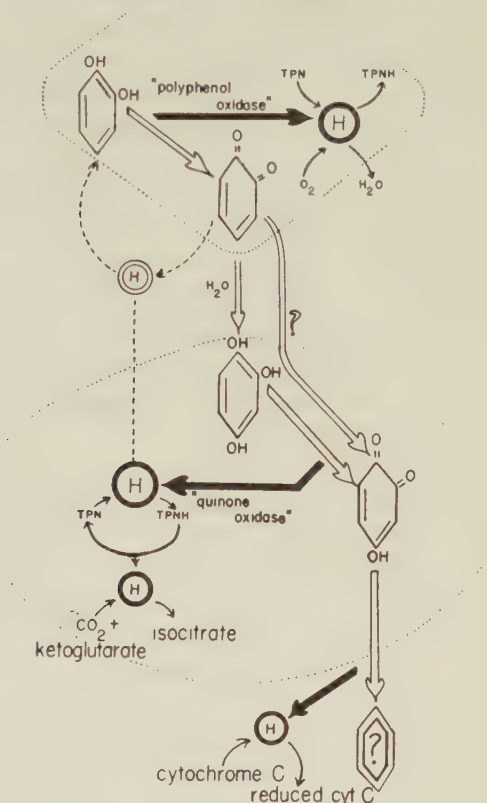


Figure 19. Schematic representation of the presumed mechanism for the enzymatic oxidation of catechol by cell-free preparations of *Blastocladiella emersonii*.

Experiments with similar reaction mixtures containing supernatant, catechol, and enzymatically-generated, colored quinones revealed that the addition of TPN, unlike the addition of cytochrome, did not cause the disappearance of the hydroxy-o-quinone; that is, although TPN was rapidly reduced (increase in absorption at 340 m μ as exemplified by results already shown in Figure 8), a simultaneous decrease in optical density at 480 m μ did not occur. In fact, the addition of TPN induced a small but reproducible, gradual increase in absorption at 480 m μ , and the effect was accentuated when ketoglutarate was present along with the TPN (ca 0.03 to 0.05 change in O.D. at 20 minutes). When reaction mixtures were boiled prior to the addition of TPN, no subsequent change in optical density occurred at either 340 or 480 m μ . It appeared once again as if TPN or ketoglutarate or both had driven the reaction leading to the formation of hydroxy-o-quinone. Thus, whereas reduction of cytochrome C was apparently geared to the non-enzymatic

oxidation of hydroxy-o-quinone, reduction of TPN was mediated enzymatically but apparently *not* driven by the further oxidation of hydroxy-o-quinone; presumably, therefore, it *was* coupled to the reaction responsible for the genesis of hydroxy-o-quinone, that is, the oxidation of o-quinone (or tri-hydroxy benzene). It is notable, incidentally, that earlier experiments (e.g. those represented in Figure 8) had already provided presumptive evidence that reduction of TPN was mediated enzymatically — experiments wherein the specificity of TPN had been demonstrated by the fact that DPN was not reduced during the oxidation of catechol by *Blastocladiella* supernatants.

Assays for other enzymatic activities. — For reasons which will be clarified in subsequent sections and in the discussion, it was necessary to establish whether or not our cell-free preparations of resistant sporangia displayed any cytochrome oxidase, succinoxidase, ketoglutarate oxidase, or isocitrate dehydrogenase activity.

The techniques used, already employed in the study of homogenates of the thin-walled plants of *B. emersonii* (Cantino and Hyatt, 8) were briefly as follows: (1), oxidation of isocitrate was followed by the coupled reduction of TPN at 340 m μ and by the coupled reduction of cytochrome C, *via* TPN, at 550 m μ (see protocol under Figure 9); (2), oxidation of succinate, by the coupled reduction of cytochrome C, in the presence of a cyanide block, at 550 m μ (0.3 ml. supernatant, 1.0 ml. 1.4×10^{-1} M phosphate, pH 7.0, 0.63 ml. water, 0.5 ml. 1.7×10^{-4} M cytochrome C, 0.075 ml. 0.006 M KCN, and 0.5 ml. 10^{-1} M succinate); (3), oxidation of ketoglutarate, by the coupled reduction of cytochrome C, *via* DPN, in the presence of a malonate and cyanide block, at 550 m μ (0.3 ml. supernatant, 1.0 ml. 1.4×10^{-1} M phosphate, pH 7.0, 0.19 ml. water, 0.5 ml. 1.7×10^{-4} M cytochrome C, 0.075 ml. 0.006 M KCN, 0.5 ml. 10^{-1} M ketoglutarate, 0.2 ml. 1.5 M malonate, and 0.24 ml. 2.5×10^{-3} M DPN); (4), oxidation of reduced cytochrome C, by measurements at 550 m μ (0.3 ml. supernatant, 0.5 ml. 1.7×10^{-4} M reduced cytochrome C in 1.4×10^{-1} M phosphate, pH 7.0, and 1.0 ml. 1.4×10^{-1} M phosphate). TPN-specific, isocitrate dehydrogenase activity was always easily detectable in our cell-free preparations (Figure 9). That the reaction was reversible was suggested by the fact that these same preparations also brought about a small but significant reduction of ketoglutarate, but only in the presence of bicarbonate (Figure 15; cf. also subsequent section). On the other hand, it was impossible to detect any ketoglutarate oxidase, succinoxidase, or cytochrome oxidase activity. Because such activity was always easily demonstrable in homogenates of freshly-harvested, thin-walled plants (Cantino and Hyatt, 8, 9), the assays were also performed on undialyzed supernatants at several different concentrations derived from freshly-harvested cultures of resistant sporangial plants. Once again, the cell-free extracts were clearly deficient in ketoglutarate oxidase and cytochrome oxidase, and they possessed only very slight (ca one-tenth of that in thin-walled plants; Cantino and Hyatt, 8) succinic dehydrogenase activity.

Identification of the products derived from ketoglutarate during the enzymatic oxidation of catechol. — As a working hypothesis, it had been

assumed that ketoglutarate stimulated the oxidation of catechol by virtue of its concomitant reduction to isocitrate *via* the TPN-specific, isocitric dehydrogenase.

To test this hypothesis further, reaction mixtures containing 5.1 ml. supernatant, 5.1 ml. 0.27 *M* catechol, 4.25 ml. 2.5×10^{-3} *M* TPN, 8.5 ml. 0.1 *M* ketoglutarate, 11.05 ml. water, and 17.0 ml. bicarbonate-phosphate buffer pH 6.8 (2.0 g. NaHCO_3 per 100 ml. 1.7×10^{-1} *M* phosphate, pH 5.7) were incubated at 30° C. Control vessels without ketoglutarate, and without catechol, were also set up. Twelve ml. aliquots of each reaction mixture were removed at zero time, and at 20, 60, and 120 minutes after addition of enzyme; they were then cleared immediately with zinc hydroxide. Organic acids were removed from these supernatants by continuous liquid-liquid ether extractions in some of the experiments; they were separated from one another by paper chromatography and distillation, characterized by their *rf* values, Duclaux constants, and certain color reactions, and other quantitative determinations as outlined in Material and Methods. The only acids recovered were ketoglutarate, lactate, and acetate, and trace quantities of isocitrate.

The results revealed clearly that lactate and acetate, in roughly equivalent amounts, and trace quantities of isocitrate, gradually accumulated in the reaction mixtures during the course of the oxidation of catechol, and that up to 40 per cent of the ketoglutarate simultaneously disappeared. No other acidic products were detected. However, the lactate and acetate accounted for only 10 to 20 per cent of the ketoglutarate metabolized. This aspect of the problem is being pursued further, but the results are presented here because of their direct bearing upon the problem under investigation. These observations, together with (1), the fact that cell-free preparations of *Blastocladiella* were devoid of ketoglutarate oxidase, cytochrome oxidase, and succinoxidase activity but did possess isocitrate dehydrogenase activity, and (2), the observed effects of ketoglutarate upon catechol oxidation, particularly in the presence of bicarbonate, provide strong support for our suggestions that ketoglutarate underwent reductive carboxylation during the oxidation of catechol.

(*Separation of the catechol-oxidizing system from the quinone-oxidizing system*). — *A priori*, it was assumed that perhaps the heterogeneous, enzymatically-active supernatants of *Blastocladiella* could be separated into (1), a fraction containing the enzyme system responsible for the oxidation of catechol to o-quinone (the latter, after hydration, being oxidized non-enzymatically by a second molecule of o-quinone to hydroxy-o-quinone) and (2), a second fraction containing the enzyme system which mediated the oxidation of tri-hydroxy benzene to hydroxy-o-quinone. If this were accomplished, then it should have been possible to demonstrate that the activity of the catechol-oxidizing system would be independent of the presence or absence of ketoglutarate, whereas either the tri-hydroxy benzene-oxidizing

Table 2. *The catechol-oxidizing activity of different fractions of cell-free preparations of Blastocladiella emersonii.* All centrifugations were carried on for 5 minutes at 1° C. Supernatants (prepared by centrifugations of homogenates at 500×G) were centrifuged successively under different centrifugal fields (see context for details). Assays for catechol-oxidizing activity were made on all supernatants and resuspended centrifugates by following the reduction of TPN at 340 mμ. Each cuvette contained: 0.3 ml. 0.27 M catechol, 0.3 ml. enzyme, 0.25 ml. 2.5×10^{-3} M TPN, 1.0 ml. bicarbonate-phosphate buffer, pH 6.8 (2.0 g. NaHCO_3 per 100 ml. 1.7×10^{-1} M phosphate, pH 5.7), and water to make 3.0 ml. Assays with each preparation were performed both in the presence and absence of 0.5 ml. of 0.1 M ketoglutarate. The basal levels of activity (change in optical density after 20 minutes, ca 0.08 as depicted in fig. 18) obtained in the absence of ketoglutarate were the same for all the supernatants.

Centrifugal field, × G	Per cent increase in activity of supernatants due to the presence of ketoglutarate ¹
500	74.0
900	71.0
1,500	95.6
3,000	192.0
10,000	173.2
20,000	141.3

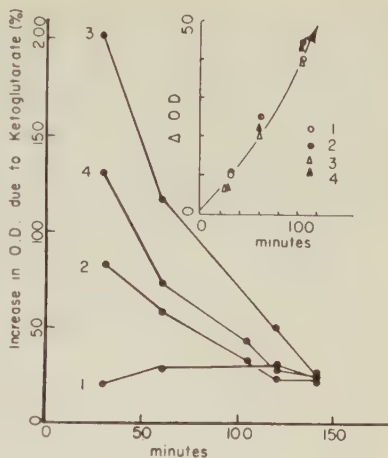
¹ The difference between increase in optical density after 20 minutes in the presence and in the absence of ketoglutarate, divided by the increase in optical density after 20 minutes in the absence of ketoglutarate; ×100.

system or the heterogeneous mixture of both enzymes would be dependent upon the presence of ketoglutarate for optimum activity. When viewed in retrospect, perhaps it would have been quite fortuitous to find that the two enzyme systems occurred in particulates of sufficiently different dimensions such that they could be separated directly by differential centrifugation. Nevertheless, attempts were made to test this possibility.

Homogenates, prepared in the usual way, were centrifuged at 500×G. The centrifugates were then resuspended and the supernatants were centrifuged once again at 900×G. The centrifugates derived therefrom were resuspended once more. Supernatants and centrifugates were separated in similar fashion following subsequent centrifugations at 1500, 3000, 10,000, and 20,000×G. Each supernatant and each resuspended centrifugate was then assayed for catechol-oxidizing activity spectrophotometrically *via* the coupled reduction of TPN at 340 mμ, both in the presence and absence of ketoglutarate.

From the results in Table 2, it is evident (1), that ketoglutarate significantly stimulated the rate at which each supernatant reduced TPN; but (2), that a maximum degree of stimulation was associated with the supernatants derived from the centrifugations at 3000×G. Assays also revealed that isocitrate dehydrogenase activity (see previous section for assay methods) was easily detectable in the supernatants but not in the resuspended centri-

Figure 20. *The time course of the effect of added ketoglutarate upon the oxidation of catechol by different fractions of acetone powders derived from supernatants, as measured by the formation of red oxidation products.* Supernatants were prepared as for Figure 8. (For preparation of acetone powders and subsequent fractionations, see context). Each vessel contained 0.6 ml. $2.7 \times 10^{-1} M$ catechol, 0.6 ml. enzyme, 2.0 ml. bicarbonate-phosphate buffer (see Table 3), and water to make 6.0 ml. Assays with each preparation were performed both in the presence and absence of $1.0 \text{ ml. } 10^{-1} M$ ketoglutarate on the Klett-Summerson colorimeter (green filter). Curves 1, 2, 3, and 4 correspond to enzyme preparations derived by fractional precipitation with 3.02, 4.54, 6.04, and 6.80 g. ammonium sulfate/10 ml. respectively (cf. context). The basal rates obtained for each preparation in the absence of added ketoglutarate are shown in the insert.



fugates. Thus, it was impossible to effect directly, a complete resolution of the two enzyme systems by differential centrifugations in fields as high as $20,000 \times G$.

However, the technique of salting out proteins is also based to some extent upon consideration of the size of protein molecules, and in a general way, the size of the precipitated protein molecule is inversely related to the concentration of salt that induces it to precipitate. Using undialyzed supernatants derived from homogenates centrifuged at $500 \times G$, very active acetone fractions were also prepared by mixing the former with 10 volumes of acetone and then resuspending the precipitates in phosphate buffer. In this case, using the phosphate-bicarbonate buffer once again, the strong stimulatory effect of ketoglutarate (average of 200 per cent increase in activity) was easily demonstrable whenever the enzyme was dialyzed overnight against phosphate. Dialysis reduced the catechol oxidizing capacity to ca one fourth or less of that for the undialyzed preparations, and the addition of ketoglutarate brought about almost complete recovery of this activity. Acetone powders, derived from undialysed supernatants, were then suspended in phosphate buffer; ammonium sulfate (3.02 g./10 ml.) was dissolved therein, and they were then centrifuged. In similar fashion, the supernatants derived therefrom were subjected to successive fractional precipitation with ammonium sulfate at 4.54, 6.04, and 6.8 g./10 ml. The precipitates were each redissolved in a volume of phosphate buffer equivalent to $1/4$ of the original volume of supernatant. These preparations were incubated at $30^\circ C$ with catechol and bicarbonate-phosphate buffer in the presence and absence of ketoglutarate; the course of the reaction was followed colorimetrically. The results (Figure 20) revealed that, in the absence of ketoglutarate, all frac-

tions displayed rather low, but fully-comparable, catechol-oxidizing activities. However, whereas the addition of ketoglutarate to these reaction mixtures induced only slight stimulation of the activity in one preparation and a somewhat greater stimulation in a second, it caused a pronounced stimulation of that in the other two fractions.

Characterization of the brown pigment. — There are apparently a variety of melanin-like pigments, and no simple, direct, and definitive methods for establishing their identity. We therefore chose, tentatively, to characterize the deep brown pigment in the walls of resistant sporangia on the basis of criteria such as those employed by Schaeffer (24) in an investigation of the brown pigments produced by a mutant strain of *Neurospora*. A dried, powdered preparation of *B. emersonii* was refluxed for 30 minutes in alkali (450 mg. powder/30 ml. 0.5 N NaOH). The brown, slightly-turbid, alkaline solution was filtered and diluted with an equal volume of water, and the absorption spectrum of the filtrate was determined between 400 and 600 m μ . The slope of the plot, log optical density vs wave length (0.00285, average of our data) was almost identical with that (0.0027, average of Schaeffer's data) reported by Schaeffer (Figure 10). Saturation with ammonium sulfate induced precipitation of a brown, flocculent precipitate. These results, along with our observations of the effects of phenylthiourea on the biosynthesis of the brown pigment *in vivo* (Cantino, 5) and the enzymatic oxidation of catechol *in vitro* (reported herein), are consistent with, and offer evidence for, the belief that the pigment is indeed melanin.

Discussion

As a result of the first strictly *in vivo* studies of *Blastocladiella emersonii* (Cantino, 3, 4), it was ascertained that developmental pathways for this organism were clearly under the control of the environment. More specifically, the presence of elevated concentrations of bicarbonate invariably induced young thalli to develop into plants bearing resistant sporangia with thick, pitted, chitinous walls impregnated with a deep-brown, presumably melanin-like pigment. In the absence of added bicarbonate, thalli developed instead into plants whose sporangia were thin-walled and papillate, free of pits, and completely devoid of the brown pigment. Thus, the causal relation between imposition of the bicarbonate "trigger mechanism" and the *in vivo* formation of brown resistant sporangia was an easily-demonstrable, established fact.

On the other hand it was a much more difficult chore to elucidate, from such *in vivo* investigations, the fundamental role that bicarbonate must have

played *within* the plant to induce and maintain the morphogenetic mechanism. Nevertheless, the data then available were generally consistent with, and led to, the hypothesis that bicarbonate exerted its effect on morphogenesis by interfering with the decarboxylation of ketoglutarate in the tricarboxylic acid cycle. Intrinsic to the hypothesis was the assumption that (1), ketoglutarate and its precursors in the cycle gradually piled up to some extent within the organism, and (2), that one or more of these intermediates must have been involved, directly or indirectly, in the initiation and maintenance of theretofore non-existent shunt mechanisms which led to the formation of the basic ingredients of a resistant sporangium such as the melanin pigments (Cantino, 3, 4).

Subsequently, the hypothesis was significantly strengthened by more definitive, direct, evidence derived from *in vitro* investigations using cell-free preparations of several different strains of Blastocladiella (Brown and Cantino, 1; Cantino, 5, 6, 7; Cantino and Hyatt, 8, 9).

Up to that time, however, no direct evidence was available for elucidating the *mechanism* by which ketoglutarate (or its precursors), which accumulated in Blastocladiella, somehow initiated and maintained the metabolic shunts which led to the synthesis of the chemical constituents found in a resistant sporangium. Believing that it would be the biosynthetic reactions leading to melanin synthesis which could be most amenable investigated in cell-free systems, it was decided (1), to look for a polyphenol oxidase in homogenates of *B. emersonii* and (2), provided such a system were found, to determine if (and if so, how) its activity was, in fact, coupled to and dependent upon the presence of ketoglutarate. The bulk of the results obtained so far, which constitutes the framework of this report, supplies some of the direct evidence needed to substantiate our thesis that the ketoglutarate which piles up as a result of the bicarbonate »trigger mechanism« does, indeed, play a vital role in the initiation and maintenance of melanin biosynthesis.

In the absence of added bicarbonate in the immediate environment, young thalli of Blastocladiella mature along the alternate developmental pathway which leads to the formation of colorless, thin-walled plants. A polyphenol oxidase system, capable of oxidizing test substrates such as tyrosine, dihydroxy-phenylalanine, and catechol, could not be detected in such thin-walled plants. On the other hand, previous investigations had already demonstrated that thin-walled plants do possess a vigorous cytochrome oxidase and a battery of enzymes capable of oxidizing almost all of the intermediates of the tricarboxylic acid cycle (Brown and Cantino, 1; Cantino, 5; Cantino and Hyatt, 8, 9).

But, following imposition of the bicarbonate »trigger-mechanism«, thalli of Blastocladiella mature *via* the developmental pathway which leads to the

formation of brown resistant sporangia. In these resistant sporangial plants, the »polyphenol oxidase« system defined above was always present and easily detected. Moreover, although the isocitric dehydrogenase activity found in thin-walled plants was similarly demonstrable in resistant sporangial plants, other enzyme systems, notably ketoglutarate oxidase, succinoxidase, and cytochrome oxidase had either disappeared or become inoperative.

Although tyrosinase does not appear to function as a terminal oxidase in some of the fungi that have been investigated from this point of view (cf. for example, Sussman and Markert, 27, on *Glomerella*), it has been suggested by others (for example, Bonner and Wildman, Streerangachar, etc.; reviewed in Dawson and Tarpley, 13) that it may do so in higher plants. The evidence now available tempts us to suggest, too, that during morphogenesis in *B. emersonii*, a newly-synthesized »polyphenol oxidase« assumes the role of terminal oxidase as the pre-existing cytochrome oxidase gradually disappears.

It seemed, then, that the presence of increased concentrations of bicarbonate within the plant not only interfered with the decarboxylation of ketoglutarate but actually brought the tricarboxylic acid cycle to a standstill. The enzyme systems normally required for the further degradation of ketoglutarate, such as ketoglutarate oxidase, succinic dehydrogenase, and the terminal cytochrome oxidase, apparently would thus have been rendered quite dispensible; the experimental data revealed that they did, indeed, disappear. On the other hand, at least one of the enzymes required for reversal of the cycle *via* reductive carboxylation of ketoglutarate, namely isocitrate dehydrogenase, was retained and was clearly functional. Apparently, the reversal of metabolism extended backward to a C_2 plus C_4 stage, because both lactate and acetate were detected as products of the reductive carboxylation of ketoglutarate.

Of utmost significance, however, was the observation that the two systems — »polyphenol oxidase« and isocitrate dehydrogenase — were interrelated and coupled to one another. The »polyphenol oxidase« system was clearly dependent upon the presence of ketoglutarate for optimum activity; ketoglutarate, in turn, appeared to function as a required co-factor by virtue of its concomitant reduction *via* a TPN-specific isocitrate dehydrogenase.

These results, therefore, provide further corroboration for our hypothesis regarding the biochemical basis for morphogenesis in *B. emersonii*; specifically, they supply direct evidence that oxidation of polyphenols (and therefore, probably melanin synthesis) is coupled to the accumulation of ketoglutarate which results from the imposition of the bicarbonate »trigger mechanism«. We wish to stress that in our view, because of the intent and purpose of our investigations, the primary significance of these observations lies in the fact that they lend themselves to a further elucidation and integra-

tion of the growth physiology and general biology of *Blastocladiella emersonii*. However, the mechanism of the oxidation of catechol, and the nature of the coupling between this oxidation and the reduction of ketoglutarate, are of interest in their own right. The following section is therefore devoted to an interpretation of the mechanism of the enzymatic oxidation of catechol by cell-free preparations of *B. emersonii*.

In most instances where polyphenol oxidase activity has been investigated in fungus homogenates (e.g. Sussman and Markert, 27, on *Glomerella*, Horowitz and Shen, 15, on *Neurospora*, and others), the enzyme has been characterized as »soluble» in that it readily appeared in supernatants and did not sediment easily under rather high centrifugal fields (e.g. up to ca $18,000\times G$). For that matter, the polyphenol oxidase activity of tissue slices and homogenates of higher plants has generally been similarly characterized, although exceptions have been reported wherein part of the activity was rather tightly bound to various kinds of particulates (cf. survey by Weir and Stocking, 29, and more recent papers such as McClendon's, 20). Most of the »polyphenol oxidase» activity of homogenates of *Blastocladiella* was firmly bound to the chitinous, melanin-impregnated, wall of the resistant sporangia; this fraction was not easily »solubilized». Part of the »polyphenol oxidase» system, however, was immediately »solubilized» following homogenization, in the sense that it could not be sedimented out in centrifugal fields as high as $20,000\times G$. In order to simplify the following discussion, the complete system (soluble and insoluble components) will be referred to as the catechol-oxidizing system.

The catechol-oxidizing system in whole homogenates readily oxidized catechol when measured *via* oxygen consumption; virtually all of this activity was lost following dialysis against phosphate buffer. Presumably, the ketoglutarate present in resistant sporangia was thus removed from these homogenates. The oxidase activity of dialyzed preparations (using tyrosine and dihydroxyphenylalanine, as well as catechol, as substrates) was regained immediately whenever ketoglutarate was also incorporated in the reaction mixture. This effect appeared to be specific in that equi-molar quantities of other acids (e.g. citrate, pyruvate, etc.) had no detectable effect upon the activity of dialyzed homogenates. What, then, was the role that ketoglutarate played in the enzymatic oxidation of catechol?

Knox and LeMay-Knox (18) demonstrated that soluble, dialyzed preparations from liver mediated the oxidation of tyrosine to acetoacetate only if ketoglutarate were incorporated in their reaction mixtures. Ketoglutarate functioned as an essential co-factor in the reaction by virtue of an initial transamination between itself and tyrosine, thus yielding p-hydroxyphenylpyruvate and glutamate. Furthermore, it was established (1), that the keto-

glutarate requirement could be replaced with pyruvate, provided that trace quantities of ketoglutarate were also present, and (2), that an additional co-factor, pyridoxal phosphate, was indispensable for the transamination between ketoglutarate and tyrosine. Rat kidney preparations appear to have the same general requirement for ketoglutarate (Crandall, 12).

Dialyzed homogenates of *Blastocladiella* displayed a requirement for ketoglutarate in the oxidation of tyrosine, dihydroxyphenylalanine, and catechol. Whether or not a transamination occurred during the oxidation of tyrosine in the presence of ketoglutarate was not ascertained. But, almost all of the investigation described herein dealt with the oxidation of catechol, and a direct transamination between ketoglutarate and catechol was obviously impossible. Besides, with catechol as substrate, (1), the need for ketoglutarate could not be replaced by pyruvate, and (2), the addition of pyridoxal phosphate to our reaction mixtures had no detectable effect upon the course of the reaction (as measured *via* oxygen consumption). It was necessary, therefore, to discover an alternate explanation for the effect of ketoglutarate upon the oxidation of catechol.

Because of our preconceived notions regarding the biochemical basis for morphogenesis in *Blastocladiella*, particularly the presumed role of bicarbonate in decelerating and perhaps even reversing the oxidative decarboxylation of ketoglutarate in the Krebs cycle, we chose the working hypothesis that oxidation of catechol could be partially coupled with the reductive carboxylation of ketoglutarate to isocitrate *via* isocitric dehydrogenase. We supposed that this type of coupling might have initiated, accelerated, and/or helped maintain the oxidation of catechol in our cell-free preparations. Inherent in such a notion, however, was the realization that it would be an essential prerequisite to demonstrate that in homogenates of resistant-sporangial plants: (1), the enzyme systems capable of oxidizing ketoglutarate *via* a terminal oxidase (for example, ketoglutarate oxidase, succinoxidase, cytochrome oxidase), which were easily demonstrable in homogenates of thin-walled plants, were either absent, weakly-functional, or impotent; and (2), the enzyme systems needed for reductive carboxylation of ketoglutarate remained intact and functional.

Spectrophotometric assays then revealed that, indeed, neither ketoglutarate, succinate, nor reduced cytochrome C were oxidized by dialyzed and undialyzed cell-free extracts of resistant sporangia. These results were quite consistent with our observations that during manometric measurements of the oxidation of catechol, oxygen uptake was not affected by: (1), arsenite, at concentrations which inhibited growth (Cantino, 3) and germination (Cantino, 5) of *Blastocladiella*, *in vivo*, and oxidation of ketoglutarate in homogenates of thin-walled plants *in vitro* (Cantino and Hyatt, 8), and (2), malonate and

cyanide, at concentrations which inhibited oxidation of succinate and cytochrome C (reduced), respectively, in homogenates of thin-walled plants (Cantino and Hyatt, 8). On the other hand, TPN-specific isocitric dehydrogenase activity was easily detected in these same homogenates by measuring the concomitant reduction of TPN at 340 m μ , or the reduction of cytochrome C, *via* carrier quantities of TPN, at 550 m μ .

Our views were further strengthened by the results of direct chemical analyses of reaction mixtures during the enzymatic oxidation of catechol in the presence of bicarbonate and ketoglutarate, wherein it was shown that some of the ketoglutarate had been converted to a mixture of lactic, acetic, and traces of isocitric acids; no other acidic metabolites were detected. Thus, in the absence of demonstrable ketoglutarate oxidase, succinoxidase, and cytochrome oxidase activity, ketoglutarate must have been reduced *via* isocitrate (presumably through a C₂ plus C₄ stage) to lactate and acetate. Up to this point, all of the available evidence was consistent with our formulations regarding the stimulatory role of ketoglutarate in the enzymatic oxidation of catechol.

Next, attempts were made to identify the electron donor (or donors) responsible for the reduction of ketoglutarate — that is, to isolate the locus for ketoglutarate reduction in the chain of reactions leading from catechol to its colored oxidation products. It was assumed *a priori*, that catechol was oxidized *via* the scheme proposed by Dawson and Tarpley (13); this involves oxidation of catechol to o-quinone, hydration of o-quinone to trihydroxy benzene, a coupled oxidation-reduction between tri-hydroxy benzene and a second molecule of o-quinone yielding hydroxy-o-quinone and regenerating a new molecule of catechol, and finally, conversion of the hydroxy-o-quinone to other products. This scheme (cf. Fig. 19) was used as a basic framework and a point of departure for further experiments.

At the start, it was necessary to assume that electrons were first transferred, directly or indirectly, to TPN because the isocitric dehydrogenase was TPN-specific. Reduction of TPN, therefore, would have to have been driven by oxidation of catechol to o-quinone (reaction 1), oxidation of trihydroxy benzene to hydroxy-o-quinone (Reaction 3), and/or further oxidation of hydroxy-o-quinone to unknown products (Reaction 4).

It would have been illogical to expect electron transfer to TPN, and thence to ketoglutarate, *solely via* reaction 1. That is, assuming that electron transfer to oxygen also occurred only at the expense of reaction 1, the addition of ketoglutarate to the reaction mixtures would have resulted in competition for electrons and therefore a decrease, rather than an increase, in oxygen consumption during the oxidation of catechol. But, it was not illogical to expect that electron transfer to ketoglutarate *via* TPN might just as easily

have occurred at the expense of reaction 3 (whereby the TPN-ketoglutarate system would compete with o-quinone for electrons), or reaction 4, or both. If the rate of reaction 1 were limited by the rate of reactions 2, or 3 or both, the presence of ketoglutarate might either accelerate oxygen consumption *via* reaction 1 or at least prevent its rate from tapering off. Attempts were then made to obtain experimental evidence which would either confirm or refute the hypothesis.

Spectrophotometric assays at 340 m μ revealed that the over-all enzymatic oxidation of catechol was, indeed, coupled to the reduction of TPN. DPN, on the other hand, was completely ineffective as an electron acceptor. These results were consistent with our speculations, and they further strengthened our ideas regarding the role of isocitric dehydrogenase in the catechol-oxidizing system; but, they still did not permit us to determine which step or steps in the reaction sequence between catechol and its final oxidation products were responsible for the reduction of TPN.

These experiments were interrupted by the puzzling observation that the rates and total quantities of TPN reduced at the expense of the oxidation of catechol were also increased significantly by the presence of ketoglutarate, and enormously by the presence of both ketoglutarate and bicarbonate, in the reaction mixtures. If TPN were being reduced *via* electron transfer from a *single* step in the catechol-hydroxy-o-quinone reaction chain, it was logical to expect that the addition of ketoglutarate would have decreased rather than increased the overall rate of reduction of TPN because of the re-utilization of part of the reduced TPN for the reductive carboxylation of ketoglutarate *via* isocitrate dehydrogenase.

But, a rational explanation would have been forthcoming if it could be shown that TPN became reduced *via* electron transfer from *two* successive oxidations, such as the oxidation of catechol to o-quinone (reaction 1), and the oxidation of tri-hydroxy benzene to hydroxy-o-quinone (reaction 3); furthermore, the explanation would have been strengthened significantly if it were shown that the locus for reaction 1 was spatially separated from that for reaction 3 (perhaps on particles of different dimensions, etc.). Under these conditions, acceleration of reaction 3 induced by the coupled reduction of ketoglutarate *via* carrier quantities of TPN might increase the rate of reduction of TPN *via* reaction 1. This scheme would also have been compatible with our observations of the stimulatory effect of ketoglutarate upon oxygen consumption.

It was then revealed that it was, indeed, quite possible to separate the enzyme complex into several fractions, each with the same basal level of catechol-oxidizing activity, but some of which were stimulated by ketoglutarate while others were not.

New experiments were therefore undertaken, making use of the fact (Tarpley, 28) that the absorption spectrum of o-quinone exhibits a well-defined peak at 390 m μ and then a rapid drop to practically zero absorption by 440 m μ , whereas hydroxy-o-quinone absorbs only slightly at 390 m μ but does absorb strongly, and exhibits a peak at, 480 to 485 m μ .

Short-time (20 minute) spectrophotometric assays revealed that: (1), during enzymatic oxidation of catechol in the presence of TPN, there invariably occurred in the first few minutes a small but reproducible rise in absorption at 390 m μ (ca 0.05 units of optical density), after which it leveled off and attained a constant value; intermittent readings at 340 m μ revealed that TPN was simultaneously reduced at a more or less constant rate (such as that delineated in Fig. 8), both before and after the increase in absorption at 390 m μ had ceased; (2), during enzymatic oxidation of catechol in the absence of TPN, reaction mixtures exhibited no change in optical density at either 340 or 390 m μ ; (3), during enzymatic oxidation of catechol in the presence of both TPN and ketoglutarate, the usual increase in rate of reduction of TPN (cf. Fig. 8) was again observed at 340 m μ , but under these conditions the initial rise in absorption at 390 m μ (obtained in the absence of ketoglutarate) could no longer be detected. These results were consistent with our suggestions that reaction 2, or 3, or both was slower than reaction 1 and thus permitted, initially, a small but significant accumulation of o-quinone, whereas in the presence of ketoglutarate, reaction 3 was maintained at a more rapid rate and no o-quinone accumulated.

When enzymatic oxidation of catechol was allowed to proceed for longer periods of time, absorption spectra of the reddish reaction mixtures were always characterized by a well-defined peak at 480 m μ , thus offering presumptive evidence that hydroxy-o-quinone did, indeed, accumulate during the oxidation of catechol. On the other hand, the complete absence of any peak at 390 m μ corroborated our belief (and that of others; e.g., Dawson and Tarpley, 13) that no appreciable quantity of o-quinone accumulated beyond that referred to above.

When similar reaction mixtures were incubated in the absence of oxygen, no oxidation of catechol occurred judging from the complete absence of colored oxidation products. But, when TPN was incorporated in these anaerobic reaction mixtures, catechol was oxidized and TPN became reduced; in the presence of oxygen, the quantity of TPN reduced was approximately half of that obtained anaerobically. Thus, the conclusion was inescapable that oxygen, an electron acceptor for reaction 1, could be effectively replaced by TPN. When both oxygen and TPN were present simultaneously, either (1), electrons from reaction 1 were transferred preferentially to oxygen while TPN remained free to accept electrons from reaction 3, or (2), oxygen

and TPN competed with one another for electrons from reaction 1, both of them receiving a certain share of them. Whichever the correct interpretation, however, it was clear that reduction of TPN was coupled to reaction 1, the oxidation of catechol to o-quinone.

When TPN was added to reaction mixtures in which colored products had been formed enzymatically from catechol, no decrease in optical density was detected at 480 m μ . Instead, a small but reproducible increase in optical density occurred at 480 m μ , the rate and magnitude of which was increased further if ketoglutarate was also added simultaneously. Thus, no evidence was obtained which would indicate that reduction of TPN could be coupled to reaction 4, the oxidation of hydroxy-o-quinone to other products.

In the aggregate, the foregoing results provided significant direct evidence for our suggestions: (1), that reduction of TPN was coupled to reaction 1, the oxidation of catechol to o-quinone; (2), that reduction of TPN was also coupled to reaction 3, the oxidation of tri-hydroxy benzene to hydroxy-o-quinone; (3), that if ketoglutarate was present to accept reduced TPN derived from reaction 3, the latter reaction was driven with increased speed and thus induced a more rapid accumulation of hydroxy-o-quinone; and (4), that reduction of TPN probably could not be coupled with the further oxidation of hydroxy-o-quinone if, indeed, the latter reaction did occur under these conditions.

It was expected that our enzymatically-generated hydroxy-o-quinone could reduce cytochrome C non-enzymatically, and that if this were so, the reaction might offer an alternative, convenient method for further characterization of certain of the steps in the enzymatic oxidation of catechol. First, it was established that cytochrome C and catechol did not react with each other non-enzymatically, but that in the presence of enzyme, reduction of cytochrome C did occur while catechol became oxidized simultaneously. Presumably, then, cytochrome C was accepting electrons generated *via* reactions 3 or 4, or both. When catechol and enzyme were incubated long enough to generate colored oxidation products, and when cytochrome was then incorporated into this reaction mixture, (1), cytochrome was reduced at a much greater rate than before, while (2), the optical density rapidly decreased simultaneously at 480 m μ , thus tending to eliminate the 480 m μ peak found in absorption spectra of such reaction mixtures. Thus, it seemed clear that hydroxy-o-quinone was oxidized (reaction 4) at the expense of a non-enzymatic reduction of cytochrome C.

The reaction between cytochrome C and hydroxy-o-quinone provided an alternative approach with which to test our conclusions regarding the role of ketoglutarate in reaction 3 of the enzymatic oxidation of catechol. When both cytochrome C and ketoglutarate were added simultaneously to pre-

viously-incubated, colored, reaction mixtures like those described above, the rate of reduction of cytochrome C was much greater than that obtained in the absence of ketoglutarate (cf. Figures 12, 13) but the rate of disappearance of the hydroxy-o-quinone (decrease in optical density at 480 m μ) was greatly decreased. If cytochrome C had been competing with ketoglutarate for electrons from reaction 3, addition of ketoglutarate should have decreased the rate of reduction of cytochrome C. But if cytochrome C were accepting electrons only from hydroxy-o-quinone, and if the presence of ketoglutarate accelerated reaction 3, the rate of reduction of cytochrome C should have been increased; this is precisely what happened. The decreased rate of removal of hydroxy-o-quinone in the presence of ketoglutarate suggests that the quinone was produced more rapidly than it could be removed by the cytochrome. Once again, these observations offer further evidence for our notions concerning the mechanism of the oxidation of catechol and the role of ketoglutarate therein.

The oxidation of o-quinone, like the analogous oxidation of »dopa-quinone«, has usually been considered to be a non-enzymatic reaction (cf. Dawson and Tarpley, 13; Raper and Wormall, 23; Sussman, 26; etc.). But sufficient presumptive and direct evidence had now accumulated to indicate that, although a simultaneous, non-enzymatic reaction could not be ruled out, the oxidation of o-quinone (as its hydration product, tri-hydroxy benzene) to hydroxy-o-quinone was also mediated enzymatically by cell-free preparations of *Blastocladiella emersonii*.

Having elucidated some of the details of the enzymatic oxidation of catechol by cell-free preparations of *Blastocladiella*, what can be said regarding the nature of the enzymes which mediated these reactions? It is generally agreed (cf. for example, review by Dawson and Tarpley, 13) that the copper enzymes (1), catalyze the direct oxidation of a substrate by oxygen; (2), do not function anaerobically in the sense that the acceptor, oxygen, can not be replaced by other acceptors such as methylene blue and similar dyes; and (3), are generally cyanide sensitive.

By way of comparison, our catechol-oxidizing complex (1), does appear to effect the (direct) oxidation of catechol by oxygen; but (2), it also effects the oxidation of catechol in the absence of oxygen provided an alternate electron acceptor such as triphosphopyridine nucleotide is available, and (3), the oxidation of catechol *via* reduction of either oxygen or TPN is not inhibited by cyanide. On these bases, our enzyme system does not appear to have much in common with the copper enzymes as a group.

More specifically, however, the enzyme tyrosinase (polyphenol oxidase) generally oxidizes tyrosine as well as a variety of other mono- and di-hydric phenols, and it is usually effectively inhibited by phenylthiourea, certain

nitrophenols, and other types of copper reagents. Laccase, on the other hand, does not oxidize the monohydric phenols, but, in addition to catechol, it also oxidizes the dihydric hydroquinone. Our catechol-oxidizing system oxidizes catechol but not hydroquinone; it is only weakly-capable of oxidizing the monohydric p-cresol. Although it is not inhibited by 2,4 dinitrophenol, 10^{-2} M phenylthiourea does completely suppress electron transfer to either oxygen or TPN.

With these facts in mind, as well as our notions regarding the mechanism of catechol oxidation by cell-free preparations of *Blastocladiella*, it is somewhat difficult to assign any one name to our catechol-oxidizing enzyme complex. But, in this connection, it is important to refer to the recent studies of Wosilait and Nason (30, 31) of a pyridine nucleotide reductase. Their report concerns a new enzyme system with the following properties: (1), the enzyme mediates a reversible reaction between p-quinone and either reduced TPN or DPN to yield the oxidized nucleotide and hydroquinone; (2), the reaction which it mediates also occurs non-enzymatically, but at a much reduced rate; (3), the reaction is not inhibited by cyanide; (4), the enzyme is rather labile at 4° C., losing over 50 per cent of its activity in about a week; and (5), the enzyme can be coupled with purified tyrosinase to mediate a reaction between reduced DPN and o-quinone to yield oxidized DPN.

Our catechol-oxidizing complex is also cyanide insensitive; however, it differs from the enzyme of Wosilait and Nason in all other essential respects: (1), it is inactive in a p-quinone-hydroquinone system; it is active only with an o-quinone-catechol system; (2), in the catechol-o-quinone system, it apparently mediates the further oxidation of o-quinone, rather than its reduction, back to catechol; (3), it is very stable at 4° C.; no loss of activity can be demonstrated in preparations maintained at this temperature for 4—5 months; (4), the reactions which it mediates do not occur non-enzymatically; and (5), that portion of the overall reaction which is coupled to the reduction of pyridine nucleotide is quite specific; DPN will not substitute for TPN.

With due consideration of all the evidence available, we therefore suggest that the functional entity in our supernatants, referred to so far as the catechol-oxidizing system, does indeed consist of several active centers; one of these is concerned with the oxidation of catechol, and another is concerned with the oxidation of o-quinone (probably as its hydrated product, tri-hydroxy benzene). We propose to refer to the latter as a TPN-specific »quinone oxidase«. Wosilait and Nason (30, 31) described an enzyme capable of effecting electron transfer between a quinone and a pyridine nucleotide. Our »quinone oxidase« appears to be a somewhat similar type of enzyme,

but with the important exception that it mediates electron transfer *via* an oxidation rather than a reduction of quinone.

The results presented in our report have provided indisputable evidence that alpha-ketoglutarate functions as a co-factor for the oxidation of catechol, tyrosine, and dihydroxyphenylalanine by cell-free preparations of resistant sporangial plants of *Blastocladiella emersonii*. The deep brown pigment in the sporangia appears to be a melanin. If it is assumed that the enzyme systems which mediate the oxidation of catechol (and tyrosine and dopa) are indeed the ones that mediate the oxidations leading to melanin synthesis *in vivo*, then the results have provided additional, strong evidence for our concepts regarding the biochemical basis for morphogenesis in *Blastocladiella*.

Summary

Additional evidence has been provided which supports our overall concepts regarding the biochemical basis for morphogenesis in the aquatic Phycomycete, *Blastocladiella emersonii*, and which, more particularly, partially elucidates the mechanism by which ketoglutarate plays a key role in the biosynthesis of melanin during morphogenesis.

Specifically, it has been demonstrated that:

(1), cell-free preparations of resistant sporangial plants of *B. emersonii* oxidize catechol, tyrosine, and dihydroxyphenylalanine;

(2), the enzymatic oxidation of catechol, which is inhibited by phenylthiourea but not by cyanide, can be coupled to the reduction of either oxygen or triphosphopyridine nucleotide, but not diphosphopyridine nucleotide:

(3), alpha-ketoglutarate is required as a co-factor for the enzymatic oxidation of catechol, apparently by virtue of a coupled reaction between (a), reduction of ketoglutarate to isocitrate, *via* a TPN-specific isocitric dehydrogenase, and (b), oxidation of o-quinone (or trihydroxy benzene) to hydroxy-o-quinone, *via* a TPN-specific »quinone oxidase«.

References

1. Brown, D. H. & Cantino, E. C.: The oxidation of malate by *Blastocladiella emersonii*. — Amer. Jour. Bot. 1955 (in press).
2. Buch, M. L., Montgomery, R. & Porter, W. L.: Identification of organic acids on paper chromatograms. — Anal. Chem. 24: 489. 1952.
3. Cantino, E. C.: Metabolism and morphogenesis in a new *Blastocladiella*. — Antonie van Leeuwenhoek. 17: 59. 1951.
4. — The biochemical nature of morphogenetic patterns in *Blastocladiella*. — Amer. Nat. 86: 399. 1952.
5. — The role of metabolism and alpha-ketoglutarate oxidase in the growth and dif-

- ferentiation of the aquatic Phycomycete *Blastocladiella emersonii*. — Trans. N.Y. Acad. Sci. (Ser. II). 15: 159. 1953.
6. — The relation between cellular metabolism and morphogenesis in *Blastocladiella*. — Delivered to A. A. A. S. symposium, Dec. 26—31, 1954, Berkeley, Calif.
 7. — Nutrition and phylogeny in the water molds in 1955; a re-evaluation. — Quart. Rev. Biol. 1955 (in press).
 8. — & Hyatt, M. T.: Further evidence for the role of the tricarboxylic acid cycle in morphogenesis in *Blastocladiella emersonii*. — Jour. Bact. 66: 712. 1953.
 9. — — Carotenoids and oxidative enzymes in the aquatic Phycomycetes *Blastocladiella* and *Rhizophlyctis*. — Amer. Jour. Bot. 40: 688. 1953.
 10. — — Phenotypic »sex» determination in the life history of a new species of *Blastocladiella*, *B. emersonii*. — Antonie van Leeuwenhoek. 19: 25. 1953.
 11. — & Horenstein, E. A.: Cytoplasmic exchange without gametic copulation in the water mold *Blastocladiella emersonii*. — Amer. Nat. 88: 143. 1954.
 12. Crandall, D. I.: L-Tyrosine oxidation in rat kidney. — Delivered at 38th annual meeting, Federation of American Societies for Experimental Biology, Atlantic City, New Jersey, 1954.
 13. Dawson, C. R. & Tarpley, W. B.: Copper oxidases (in the Enzymes, Vol. II, Part I., pp. 454. Academic Press, Inc. N.Y. 1951.
 14. Goddard, D. R. & Holden, C.: Cytochrome oxidase in the potato tuber. — Arch. Biochem. 27: 41. 1950.
 15. Horowitz, N. H. & Shen, S.: *Neurospora* tyrosinase. — Jour. Biol. Chem. 197: 513. 1952.
 16. Koenemann, R. H.: A modification of the Miller-Muntz method for colorimetric determination of lactic acid. — Jour. Biol. Chem. 135: 105. 1940.
 17. Koepsell, H. J. & Sharpe, E. S.: Microdetermination of pyruvic and alpha-ketoglutaric acids. — Arch. Biochem. Biophys. 38: 443. 1952.
 18. Knox, W. E. & LeMay-Knox, M.: The oxidation in liver of L-tyrosine to acetoacetate through p-hydroxyphenylpyruvate and homogentisic acid. — Biochem. Jour. 49: 686. 1951.
 19. Lugg, J. W. H. & Overell, B. T.: »One-» and »two-dimensional» partition chromatographic separations of organic acids on an inert sheet support. — Austral. Jour. Sci. Res. (Ser. A — Physical Sci.). 1: 98. 1948.
 20. McClendon, J. H.: The intracellular localization of enzymes in tobacco leaves. II. Cytochrome oxidase, catalase, and polyphenoloxidase. — Amer. Jour. Bot. 40: 260. 1953.
 21. Miller, W. H., Mallette, M. F., Roth, L. J. & Dawson, C. R.: A new method for the measurement of tyrosinase catecholase activity. II. Catecholase activity based on the initial reaction velocity. — Jour. Amer. Chem. Soc. 66: 514. 1944.
 22. Miller, B. F. & Muntz, J. A.: A method for the estimation of ultramicro quantities of lactic acid. — Jour. Biol. Chem. 126: 413. 1938.
 23. Raper, H. S. & Wormall, A.: The tyrosinase-tyrosine reaction. — Biochem. J. 17: 454. 1923.
 24. Schaeffer, P.: A black mutant of *Neurospora crassa*. Mode of action of the mutant allele and action of light on melanogenesis. — Arch. Biochem. Biophys. 47: 359. 1953.
 25. Schmall, M., Pifer, C. W. & Wollish, E. G.: Extraction and determination of salts of organic bases and acids. — Anal. Chem. 24: 1446. 1952.

26. Sussman, A. S.: The functions of tyrosinase in insects. — *Quart. Rev. Biol.* 24:328. 1949.
27. — & Markert, C. L.: The development of tyrosinase and cytochrome oxidase activity in mutants of *Glomerella cingulata*. — *Arch. Biochem. Biophys.* 45:31. 1953.
28. Tarpley, W. B. Jr.: On the mechanism of the catechol-tyrosinase reaction. Spectrophotometric studies. Dissertation, Columbia University, N.Y. 1951.
29. Weier, T. E. & Stocking, C. R.: The chloroplast: structure, inheritance and enzymology. — *Bot. Rev.* 18:14. 1952.
30. Wosilait, W. D. & Nason, A.: Pyridine nucleotide-quinone reductase. I. Purification and properties of the enzyme from pea seeds. — *Jour. Biol. Chem.* 206:255. 1954.
31. — — Pyridine nucleotide-quinone reductase. II. Role in electron transport. — *Jour. Biol. Chem.* 206:271. 1954.

Studies on the Photoperiodism of *Kalanchoë Blossfeldiana*

I. Effect of Age on Response to Short-Day Treatment

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Introduction

Various investigators showed that the degree of response to photoperiodic induction depends on the age of the plant specimen used; e.g. Borthwick and Parker (1938) using Biloxi soy bean, Wóycicki (1939) using *Chrysanthemum*, and Harder and von Witsch (1940/41 a and 1942) using *Kalanchoë Blossfeldiana*. In their earlier paper, Harder and von Witsch found that: the 3 months old plants flowered after exposure to short-days of 6 or 9 hours light per day; the 5 months old plants flowered under short-days of 6, 9 and 12 hours light per day, while the 7 months old plants flowered even under long-day conditions with more than 12 hours light per day. The authors concluded from these results that *K. Blossfeldiana* was a typical short-day plant when young, but it became day-neutral and flowered under all day lengths when it got old. In their later paper (1942), Harder and von Witsch used plants which just showed above the surface of the soil as well as plants 1, 2, 3, 4, 8, 12, 17, and 24 weeks old. They found that the plants which were 8 weeks old or younger did not flower after photoperiodic treatment whether given short or long-days: the 12 and 24 weeks old plants flowered after being exposed to 7 short-day cycles of 9 hours light per day; while the 17 weeks old plants flowered after exposure to 4 or 7 short-day cycles of 9 hours light per day. None of the plants flowered under long-day conditions, i.e. when the light period exceeded 12 hours per day. The authors added that their earlier conclusion that old *K. Blossfeldiana* plants were

day-neutral was erroneously conceived through an overlooked exposure of the plants to cycles of 12 hours light per day at an early stage of their development. At that time the authors used to consider a cycle of 12 hours light per day as long-day. Nevertheless, Harder and von Witsch showed clearly that the response of *K. Blossfeldiana* to photoperiodism depends on the age of the plant.

There is already a fairly large number of publications by Harder's school on the subject of photoperiodism of *K. Blossfeldiana*; but in every case, the plants used were about 6 months old. The present investigation was, accordingly, planned to study the response to short-day treatment of plants more than 6 months old.

Material and Methods

The plants used in this study were raised from two different sowings, one made on 25.1.1950 and the other on 25.6.1950. At the time when the experiments were started (3.5.1951) the plants were, accordingly, 66 and 45 weeks old respectively; the former group shall be called the old plants and the latter the young plants hereafter. All the plants were kept unbranched by removing the axillary buds as soon as they began to sprout. This procedure made the spotting of flowering rather easy since it became confined to the apical bud.

Since it has been established by Harder and his coworkers (Harder 1948) that the optimum photoperiodic induction was conceived by *K. Blossfeldiana* when the plants were exposed to short-day treatment of cycles with 8—9 hours light per day, the cycles used in the present investigation were 9 hours light and 15 hours dark. Before the short-day treatment, all the plants were kept under long-day conditions to prevent them from flowering. This was secured by supplementary electric light switched on at sunset to make the light period not shorter than 16 hours per day; or by interrupting the long dark night by an hour of electric light switched on after midnight according to Harder and Bode (1943). Electric light was supplied by 500 watt incandescent lamps held at a distance of appr. 1 meter from the plant apices. For the short-day treatment the plants were kept under a light-tight tent from 6 p.m. to 9 a.m.; and for the rest of the day they were exposed to natural day light. After the short-day treatment the plants were exposed to natural light conditions prevailing at that time; and as it was then summer, the light period was not less than 14—15 hours per day. The study was carried out in a shaded greenhouse warmed up during the cool nights by hot-air radiators. This maintained the temperature at about 20° C; but since there was no thermoregulating installation in the greenhouse, the temperature fluctuated between 15 and 25° C on a few occasions.

The young plants were 15—20 cm high and carried about 15 pairs of leaves each on 3.5.1951. The uppermost 9 pairs of leaves were left on every plant while all other leaves were cut off. The plants were then divided into 9 similar groups of 10 plants. Every group was given a certain number of short-day cycles, namely 0, 4, 5, 6, 7, 10, 15, 20 or 30 short-days.

The old plants on the other hand were 30—40 cm high and carried about 25 pairs of leaves each on 3.5.1951. They were divided into two batches as follows:

1) Old plants with young leaves: in which case the uppermost 9 pair of leaves only were left on every plant while all other leaves were removed. The plants were divided into 9 similar groups of 10 plants each and every group received a certain number of short-day cycles, namely 0, 4, 5, 6, 7, 10, 15, 20 or 30 as in the case of the young plants.

2) Old plants with young and old leaves: in which case all the leaves, except the yellow or damaged lowermost ones, were left on the plant. They were then divided into 6 groups of 10 plants each, and every group received a certain number of short-day cycles, namely 0, 5, 7, 10, 15 or 30 short-days.

Observations and Results

The following features were proved by Harder and his coworkers (Harder 1948) to provide quantitative indications of the response of *K. Blossfeldiana* to short-day treatment:

1. Lapse of time between the beginning of short-day treatment and the appearance of the first flower primordia; then the opening of the first flower bud.
2. The total number of flowers borne on the mature inflorescence.
3. The degree of phyllody (leafy character) of bracts, and the reduction in the structure of the individual flowers.
4. Size of leaves and their degree of succulence.
5. Length of petioles and internodes.

The first of these features is in fact the more important, and sets in long before the other features. In view of the rather short time allowed for the experiments described here, it was not possible to record all the features thoroughly. More emphasis was, therefore, put on the first of these features which was recorded in every case. The setting of flowering was acknowledged when three minute primordia, visible to the naked eye, appeared in place of the normal vegetative apical bud. The lapse of time between the beginning of short-day induction and the setting of flowering is given in figure 1.

The results of these experiments lead to the following conclusions:

1. Neither the 45 nor the 66 weeks old *K. Blossfeldiana* plants flowered under long-day conditions, nor did they flower after receiving 4 short-day cycles.
2. The 45 weeks old plants flowered after receiving 5 short-day cycles or more, and the response increased — i.e. the lapse of time between beginning of induction and appearance of flower primordia decreased — when the

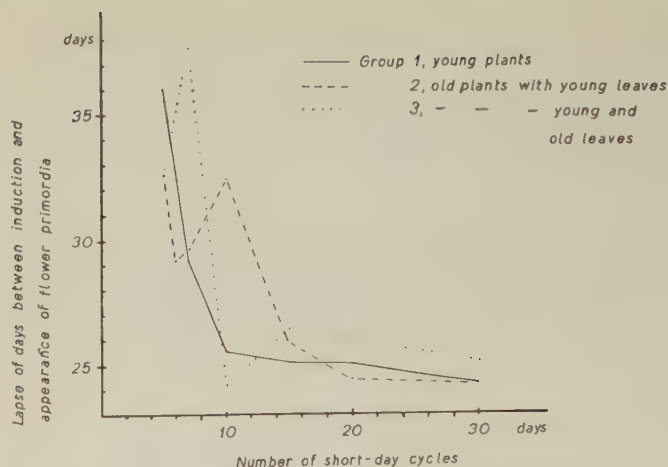


Figure 1. Effect of plant age on the response of *K. Blossfeldiana* to short-day treatment.

number of short-day cycles was increased. The increase in response was regular and proportionate with the dose of short-days up to 10 cycles. With doses more than 10 short-day cycles there was no appreciable increase in the photoperiodic response.

3. The 66 weeks old plants, whether carrying young leaves only or young and old leaves together, showed an increase in response to short-day treatment as a result of the increase in the dose of short-day cycles. However, the increasing response exhibited by both groups was very irregular and, in fact, showed marked set backs when the short-day dose was less than 15 cycles. With doses larger than 15 short-day cycles, the 66 weeks old plants flowered as readily as the young plants.

Although the inflorescences were not completely formed at the time when the study was discontinued, there was a very marked phyllody of the bracts of the 5-short-day induced inflorescences. This phyllody was less marked among the 6- and 7-short-day induced inflorescences, and disappeared completely from the 10-short-day induced inflorescences. This change in phyllody can be easily recognized from the photographs of figure 2.

Discussion

Harder and von Witsch's studies (1940/41 and 1942) were concerned primarily with the effect of plant age on the response to short-day treatment; yet the authors did not enter in these two papers any record of the lapse of time between the beginning of short-day induction and the appearance



Figure 2. *Kalanchoë Blossfeldiana* inflorescences borne on plants receiving various doses of short-day cycles:

A: 5 short-day cycles showing high degree of phyllody of bracts.

B: 7 short-day cycles showing little phyllody of bracts.

C: 10 short-day cycles showing no phyllody of bracts.

of flower primordia; they were interested in finding out whether the plants flowered at all or not. Consequently they recorded the percentage of plants flowering, and this was done when the inflorescences were already at a relatively advanced stage of development. It is not possible, therefore, to make any useful comparison between the photoperiodic reaction of their young plants (24 weeks old and younger) and that of the older plants (45 and 66 weeks) of the present investigation.

However, reviewing the other papers published by Harder's school on various aspects of photoperiodism of *K. Blossfeldiana* it was possible to collect some data showing the responses of plants at various ages to various short-day doses. These data are given in table 1. Care was taken to select and enter in this table data obtained from plants grown and maintained under conditions similar to the conditions under which the plants of the present investigation were handled.

The table is rather interesting in revealing the responses of *K. Blossfeldiana* plants of various ages to similar as well as to different short-day doses. Plants less than 11 weeks old did not flower at all, even after being given a fairly large number of short-day cycles. Plants between 12 and 24 weeks of age showed a positive response when given 7 short-day cycles; but they did not flower when given smaller doses of short-day cycles. At the age of 26 weeks it seems that the plants attained the zenith of their physiologic activity as far as flowering in response to short-day induction is concerned; for at this

Table 1. *Flowering responses of K. Blossfeldiana of various ages to short-day treatment of 9 hours light per day.*

Reference	Age in weeks	Number of short-day cycles	Response	Days between induction and flowering	Remarks
Harder and v. Witsch, 1940/41 a	8	18 48	—v 20 % +v	not recorded	
Harder and v. Witsch, 1940/41 b	21	until flowering	+v	31	3 or 5 leaves only induced
Harder and v. Witsch, 1942	0—8	0 to 42	—v		
	11	1, 2, 4 or 8	—v		
	12	1, 2, 4 or 7	—, —, —, +	not recorded	
	17	1, 2, 4 or 7	—, —, +, +	not recorded	
	24	1, 2, 4 or 7	—, —, —, +	44	
Harder and Gümmer, 1949/50 ..	26	until flowering	+v	29	
Harder, 1953	26	2	+v	24.8	18 h light/day as long-day following short-day treatment
		8	+v	20.7	
		16	+v	13.6	
Harder and Bünsow, 1954	31	2	+v	43	16 h light/day as long-day following short-day treatment
		3	+v	37	
		4	+v	36	
		8	+v	27	

N.B. —, or —v means negative flowering response, and +, or +v means positive flowering response.

age a stimulus as little as 2 short-day cycles was sufficient to induce flowering within 25 days, and larger doses of short-day induced flowering within shorter time still. The 31 weeks old plants were obviously less active in response; they required 43 days to flower after a dose of 2 short-day cycles. The 45 and 66 weeks old plants (present investigation) were still less active than the younger ones, for they did not flower at all when given 4 short-day cycles. Furthermore, they flowered after appr. 25 days when given more than 10 short-day cycles whereas the 26 weeks old plants flowered within 25 days when given 2 short-day cycles only.

These observations suggest a relationship between the age of the plant and its readiness to flower in response to short-day induction which may be represented graphically by a skewed curve, with the 26 weeks old plants occupying the turning point of the curve, the younger plants represented by the more steep side of the skew, and the older plants represented by the less steep side of the skew.

Summary

45 and 66 weeks old plants of *Kalanchoë Blossfeldiana* did not flower under long-day conditions nor when given 4 short-day cycles of 9 hours light per day.

5 to 7 short-day cycles induced flowering in both groups but with marked phyllody of the bracts; 10 short-day cycles and more induced flowering with normal inflorescences.

By increasing the dose of short-day cycles there was a regular increase in response in terms of earlier flowering of the 45 weeks old plants; this feature was irregularly displayed by the 66 weeks old plants.

Comparing these results with others obtained by Harder's school it was found that the 26 weeks old plants are probably the most active ones in response to short-day flower induction, and that younger as well as older plants are less active.

Sincere thanks are due to Prof. R. Harder for suggestion and revision of text, and for allowing me to conduct the experiments in his institute at Göttingen (West Germany).

References

- Borthwick, H. A. & Parker, N. W.: Effectiveness of photoperiodic treatment of plants of different age. — *Bot. Gaz.* 100: 245. 1938.
- Harder, R.: Vegetative and reproductive development of *Kalanchoë Blossfeldiana* as influenced by photoperiodism. — *Symp. Soc. Exp. Biol.* II. Growth: 117. 1948.
- Über den Einfluss der Tageslänge nach der photoperiodischen Induktion auf die Infloreszenzen von *Kalanchoë Blossfeldiana*. — *Planta* 42: 19. 1953.
- & Bode, O.: Über die Wirkung von Zwischenbelichtungen während der Dunkelperiode auf das Blühen, die Verlaubung und die Blattsukkulenz bei der Kurztagspflanze *Kalanchoë Blossfeldiana*. — *Planta* 33: 469. 1943.
- & Bünsow, R.: Über die Wirkung der Tageslänge vor der Kurztaginduktion auf die Blütenbildung von *Kalanchoë Blossfeldiana*. — *Planta* 43: 315. 1954.
- & Gümmer, G.: Über die Blütenbildung von *Kalanchoë Blossfeldiana* in verschiedenen Licht-Dunkelrhythmen. — *Planta* 37: 12. 1949/50.
- & von Witsch, H.: Über die Bedeutung des Alters für die photoperiodische Reaktion von *Kalanchoë Blossfeldiana*. — *Planta* 31: 192. 1940/41 a.
- Über die Einwirkung von Kurztagsblättern auf in Langtag befindliche Blätter und Stengelteile der gleichen Pflanze. Untersuchungen zur Frage nach einem formbeeinflussenden Wirkstoff. — *Planta* 31: 523. 1940/41 b.
- — Weitere Untersuchungen über die Veränderung der photoperiodischen Reaktion von *Kalanchoë Blossfeldiana* mit zunehmendem Alter der Pflanzen. — *Planta* 32: 547. 1942.
- Wóycicki, St.: Über Photoperiodizität bei Chrysanthemen. — *Gartenbauwiss.* 13: 194. 1939.

Studies on the Photoperiodism of *Kalanchoë Blossfeldiana*

II. A. Flowering of Dutch Variety

B. Flowering of Cuttings Under Short-Days

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A. Flowering of the Dutch variety of *K. Blossfeldiana*

K. Blossfeldiana, Holländische Rasse, though usually available at the »Pflanzenphysiologisches Institut, Göttingen», was never used in a major study like the other common variety. It is known to be a short-day plant, but the exact length of the light period per day beyond which no flowering could be induced was not yet determined. The experiments described below were conducted to tackle this point.

Material and method

The plants used in these experiments were raised from a sowing made on 9.9.1950 and then grown singly in small flower pots; they were about 35 weeks old at the beginning of the study. At that time each plant carried about 10 pairs of leaves, the apical 6 of which were left intact while all the other leaves below them were removed. Branching of the main axis was prevented by removing the axillary buds as soon as they started to sprout. Selected and matched plants were grouped in 11 groups of 7 plants each. Before short-day induction, the plants were kept under long-day conditions by supplementary electric light switched on at sunset to maintain a light period not shorter than 15 hours per day. After short-day treatment the plants were transferred to the natural long-day conditions prevailing at that time since it was then summer.

The photoperiodic treatment was effected by exposing every group of plants to a definite light/dark combination within the normal 24-hours cycle. The various light

durations to which the different groups of plants were exposed were: 9, 9 ¹/₂, 10, 10 ¹/₂, 11, 11 ¹/₂, 12, 12 ¹/₂, 13, 13 ¹/₂, and 14 hours light per day. Within this wide range of illuminations, therefore, some groups of plants received definite short-day cycles while others received definite long-day cycles. The dark treatment was effected by covering every group of plants separately by a light-tight enclosure made from thick black paper stretched over a wooden frame approx. 50×50×50 cm. All the plants were covered by these light-tight enclosures at 11 p.m. Starting at 9 a.m. the following day, the first enclosure was lifted up to expose a group of plants to natural day light; half an hour later, the second group of plants was exposed, and so on every half hour another group was exposed until 2 p.m. Between sunset and 11 p.m., electric light was supplied to the plants from two 500 watt lamps at a distance of 100—120 cm. from the plant apices.

Since the main object of the study was to determine the day length beyond which no flowering could be induced, it was necessary to eliminate the other important factor known to affect flowering, namely, the number of photoperiodic cycles applied. This was attained by exposing every group of plants to 20 cycles, since according to previous experience with the other variety of *K. Blossfeldiana* it was found that this number of cycles was more than adequate to induce flowering.

The experiments were conducted in a greenhouse warmed up during the cool nights by hot-air radiators. The temperature was maintained at about 20° C, but it fluctuated between 15 and 25° C on a few occasions since there was no thermo-regulation in the greenhouse.

Results and comments

The results of the study are summarized in table 1.

The figures show that day lengths of 9 ¹/₂, 10, 10 ¹/₂ and 11 hours were equally effective in inducing flowering within 28 to 30 days; the slight differences between the average lapse of days till flowering under these day lengths are statistically nonsignificant. When the plants were given 9 hours light per day, they showed a slight delay of flower initiation (31.6 days). The delay is statistically significant when the figure is compared with that of plants under 9 ¹/₂ or 10 ¹/₂ hours light per day, but it is nonsignificant when compared with that of plants given 10 or 11 hours light per day. When the light period was increased to 11 ¹/₂ hours per day, there appeared a very marked delay of flowering (42.2 days) which is statistically significant when compared with the time of flowering under all the other shorter durations of light period. When the light period was increased to 12 hours or more, there was no flowering at all.

These results show clearly that the Dutch variety of *K. Blossfeldiana* is definitely a short-day plant at the age of 35 weeks. The critical light duration beyond which no flowering could be induced is 11 ¹/₂ hours light per day. In this respect, the Dutch variety differs from the common variety since, according to Harder (1953) a light period of 12 hours per day could be regarded as short-day and induced flowering in the common variety of *K.*

Table 1. *Flowering of the Dutch variety of K. Blossfeldiana after photoperiodic induction with 20 cycles of various light durations within the 24-hours.*

Group No.	Hours of light per day	Percentage of plants flowering	Lapse of time between induction and appearance of flower primordia	
			Mean of 7 plants	Standard error of the mean
1	9	100	31.6 days	1 days
2	9 1/2	100	28 »	0.69 »
3	10	100	29.9 »	0.94 »
4	10 1/2	100	27.7 »	0.65 »
5	11	100	30 »	1.24 »
6	11 1/2	100	42.5 »	1.85 »
7	12	zero	—	—
8	12 1/2	zero	—	—
9	13	zero	—	—
10	13 1/2	zero	—	—
11	14	zero	—	—

Blossfeldiana. Harders statement is endorsed by the earlier findings by Harder and von Witsch (1940/41 and 1942) that the common *K. Blossfeldiana* plants 21 and 18 weeks old respectively flowered under cycles of 12 hours light per day.

B. Flowering of *K. Blossfeldiana* cuttings under short-day conditions

During the several years of experimentation with *K. Blossfeldiana*, Harder and his coworkers raised their plants from seed. Like most other members of the *Crassulaceae*, the plant can be easily propagated by cuttings. Since there has been no attempt to study the behaviour of cuttings towards short-day treatment, the present investigation was conducted to enquire into this matter.

Material and method

The cuttings were obtained from a stock of plants approx. 66 weeks old and 30—40 cm. high, grown under long-day conditions. The top half of the plant with only the terminal 9 pairs of leaves left on it was used as the cutting. As soon as it was severed from the parent, it was planted in a separate flower pot by pushing approx. 4 cm of the stem down into the soil. The long-day treatments before and after the short-day inductions, and the conditions of the greenhouse, where the experiments were conducted, were exactly the same as described under part A. All the axillary buds were cut off as soon as they started to sprout.

The cuttings were matched and grouped into 17 identical groups of 8—10 plants each. They were treated in the following manner: Group 1 was kept as control and received no short-day induction. Groups 2, 3, 4, 5 and 6 received 5 short-day

cycles each after 0, 10, 20, 31 and 40 days from planting the cuttings, respectively. Groups 7, 8, 9, 10 and 11 received 10 short-day cycles each after 0, 10, 20, 31 and 40 days from planting the cuttings, respectively. Groups 12, 13, 14, 15 and 16 received 30 short-day cycles each after 0, 10, 20, 31 and 40 days from planting the cuttings, respectively. Group 17 received 15 short-day cycles after 50 days from planting the cuttings. By short-day it is meant an exposure of the plants to 9 hours of natural diffuse light per day; during the remaining 15 hours, the plants were kept under a light-tight tent.

Observations and results

The plants were examined regularly every day to spot out and record the first signs of flowering which were acknowledged by the appearance of three little primordia visible to the naked eye in place of the normal vegetative apical bud. Although the cuttings were watered regularly every day and the atmosphere of the greenhouse was maintained humid throughout the experiment, the leaves began to show marked loss of turgidity a week after cutting and planting. Approximately 10 days after planting, the edges of the leaves became red in colour, apparently due to the accumulation of anthocyanin. During the third week, the leaves began to regain their turgidity and by the fourth week the red colour at the edge of the leaves began to disappear. Few plants left aside were pulled out occasionally to examine the development of roots, and it was noticed that no roots appeared on the buried part of the stem before the fourth week after cutting and planting.

The responses of the cuttings to the photoperiodic treatments are summarized in table 2.

In part I of these studies (p. 223) it was shown that the 66 weeks old *K. Blossfeldiana* responded by 100 per cent flowering to 5, 10 or 15 short-day cycles, and that the flower primordia appeared after 32.6, 24.2 and 26.4 days respectively. The cuttings used in the present investigation were, as stated above, taken from plants 66 weeks old. There is, however, a marked difference between the responses of the cuttings and of the intact plants.

Considering the response to 5 short-day cycles, it is obvious from table 2 that the stimulus did not induce any flowering until a month has elapsed after cutting (group 5); and even then only 40 per cent of the plants flowered, and the flowering was markedly delayed (41.5 days compared with 32.6 days for intact plants). When 40 days have elapsed after cutting (group 6), the plants flowered within the usual time, though only 62.5 per cent of them did flower.

Considering the case of 10 short-day cycles, it is obvious from table 2 that when the stimulus was applied immediately after planting the cuttings (group 7), it induced only 30 per cent of the plants to flower, and even then the flower initiation was markedly delayed to 53.7 days compared with 24.2

Table 2. Flowering of *K. Blossfeldiana* cuttings.

Group No.	Number of short-day cycles applied	Lapse of time bet. planting and short-day induction	Percentage of plants flowering	Lapse of time between induction and appearance of flower primordia
1	0	—	zero	—
2	5	0 days	zero	—
3	5	10 »	zero	—
4	5	20 »	zero	—
5	5	31 »	40	41.5 days
6	5	40 »	62.5	32.6 »
7	10	0 »	30	53.7 »
8	10	10 »	zero	—
9	10	20 »	60	38.8 »
10	10	31 »	89	33.5 »
11	10	40 »	87.5	25 »
12	30	0 »	100	32.1 »
13	30	10 »	100	32.7 »
14	30	20 »	100	26.9 »
15	30	31 »	100	28.4 »
16	30	40 »	100	23.3 »
17	15	50 »	100	20.5 »

days recorded in the case of the intact plants. Yet when the same stimulus of 10 short-day cycles was given 10 days after planting the cuttings (group 8), none of the plants flowered at all. When 20 days have elapsed after planting the cuttings (group 9), the plants showed a regain of their positive response to short-day induction; the response was still better when the stimulus was applied after 31 or 40 days following planting the cuttings (groups 10 and 11).

Considering the response to 30 short-day cycles, the results show that the stimulus was quite adequate to induce 100 per cent flowering every time it was applied. However, the effectiveness of the stimulus was checked back slightly when applied immediately after planting or 10 days later as indicated by the slight delay of flower initiation (groups 12 and 13).

When short-day treatment was applied 50 days after planting the cuttings, the response was 100 per cent flowering within 20 days from induction. As stated above, the intact plants flowered after 26.4 days when given 15 short-day cycles. Obviously, the cuttings in this case were more responsive to the photoperiodic induction than were the intact plants; however, such a conclusion could not be passed before further detailed investigation.

Figures 1 A and 1 B show clearly the inter-relations between the number of short-day cycles, the lapse of time after planting the cuttings and the setting of flowering.

Figure 1 A shows that at any time after planting the cuttings, the more the number of short-day cycles given to a group of plants, the more the number of plants flowering within this group. An only exception to this

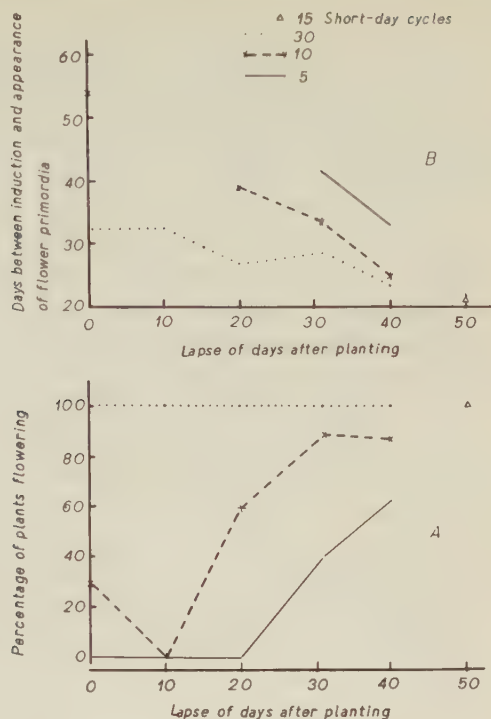


Figure 1 A and B. Response of *K. Blossfeldiana* cuttings to short-day treatment.

generalization is the case of 10 short-day cycles applied 10 days after planting the cuttings. In figure 1 B it is obvious that every curve declines towards the base line as it proceeds from left to right. This indicates that the plants flowered more readily when the stimulus was applied at a later time after planting the cuttings.

Discussion

Of the factors considered to cause a delay of flower initiation after short-day treatment of *K. Blossfeldiana*, two have been thoroughly investigated:

1. According to Harder and Lösing (1946) the decrease of the water content of the leaves proved to cause a marked delay of flowering after short-day induction.
2. According to von Denffer (1940/41) nitrogen deficiency caused a similar delay of flowering.

It is quite probable that the delay of flower initiation in the case of the cuttings was due to one or both factors. This assumption is based in the two

observations already stated above, namely, that the plants did not develop any roots until the fourth week after planting the cuttings, a feature which suggests a limited absorption of mineral salts from the soil; and second, the loss of turgidity which became more obvious during the second week after planting the cuttings.

The failure of the plants to respond to 10 short-day cycles applied 10 days after planting the cuttings is of particular interest (group 8), since the same stimulus proved effective when applied immediately after cutting (group 7). Judging by the external features of the cuttings it is not irrelevant to conclude that during the second and third weeks following cutting, the plants suffered the worse effects that could be expected as a result of the operation. A photoperiodic stimulus applied during this period was bound, therefore, to induce little or no flowering effect. The behaviour of group 7 suggests that when 10 short-day cycles were given to the plants immediately after cutting, the stimulus induced a positive flowering effect which was retained within the plant during the period of physiologic set back which resulted from the operation.

The behaviour of groups 16 and 17, i.e. cuttings given 30 and 15 short-day cycles after 40 and 50 days from planting, respectively, suggests that cuttings may even flower more readily (after 23.3 and 20.5 days) than intact plants which flowered after 24.2 and 25.6 days when given 30 and 15 short-day cycles respectively. However, this conclusion requires further confirmatory studies.

Generally speaking, therefore, it can be said that the cutting operation caused a slackening of the physiologic activity of the plant. This slackening was evidenced in the delay of flower initiation when a weak (5 short-day cycles) or a moderate (10 short-day cycles) photoperiodic stimulus was given to the cuttings. The flower initiation was markedly hastened when the new roots appeared on the cuttings.

Summary

The Dutch variety of *Kalanchoë Blossfeldiana* flowered under short-day conditions only; the critical day length beyond which no flowering could be induced is 11 1/2 hours light per day.

Cuttings of the common variety of *K. Blossfeldiana* were given 5, 10 or 30 short-day cycles of 9 hours light per day immediately after planting. The same treatment was repeated on other cuttings 10, 20, 31 and 40 days after planting. The cuttings showed a marked delay in response to the photoperiodic stimuli specially when the stimulus was applied during the second or third week following planting.

The delay in photoperiodic response may be due to a deficiency of mineral salt supply, and probably nitrogen in particular. It may be equally due to a decrease in the water content of the plant.

Sincere thanks are due to Prof. R. Harder for suggestion and for revision of text, and for allowing me to carry out the investigation in his institute at Göttingen (West Germany).

References

- von Denffer, D.: Über die Wechselbeziehungen zwischen Stickstoffbedürfnis und photoperiodischer Reaktion bei einigen Lang- und Kurztagpflanzen. — *Planta*. 31: 418. 1940/41.
- Harder, R.: Über den Einfluss der Tageslänge nach der photoperiodischen Induktion auf die Infloreszenzen von *Kalanchoë Blossfeldiana*. — *Planta*. 42: 19. 1953.
- & Lösing, I.: Über Blütenbildung und Sukkulenz bei *Kalanchoë Blossfeldiana* bei hohen Salzkonzentrationen und Bodentrockenheit. — *Nachr. Ges. Wiss. Göttingen, Math.-Phys. Kl.*: 12. 1946.
- & von Witsch, H.: Über die Bedeutung des Alters für die photoperiodische Reaktion von *Kalanchoë Blossfeldiana*. — *Planta*. 31: 192. 1940/41.
- — Weitere Untersuchungen über die Veränderung der photoperiodischen Reaktion von *Kalanchoë Blossfeldiana* mit zunehmenden Alter der Pflanzen. — *Planta*. 32: 547. 1942.

The Continuous Culture of Excised Rye Roots

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Introduction

Roots of monocotyledonous plants were used in some of the earliest attempts to culture excised roots (Kotte 1922 a, b; Robbins 1922 a, b), but hitherto there has appeared no well substantiated report of their successful culture for long periods.

Robbins (1922 a, b) and Robbins and Maneval (1924) clearly showed that the growth of excised maize roots was improved by the addition of autolysed yeast or peptone to the culture medium and by exposure of the cultures to diffuse daylight. However, with each subculture, the growth rate declined and the diameter of the roots decreased. Individual roots differed in the rate at which their growth declined but ultimately the growth of all roots ceased. Robbins and White (1936, 1937), Fiedler (1936), and Almestrand (1949, 1950 a, b, 1951), starting with media capable of supporting the continuous growth of excised roots of tomato or pea have studied in detail the response of a number of different excised cereal roots to changes in the composition of the medium. These workers have altered the nature and concentration of the carbohydrate, inorganic and vitamin constituents of the basic medium and have supplemented it by additions of amino-acids, auxins and a wide range of complex supplements (yeast extracts, protein hydrolysates, endosperm extracts, etc.). Temporary enhancement of the rate of root growth has resulted from such alterations in the composition of the culture medium; but growth rate always declined and eventually ceased whether the roots were subcultured or transferred intact at intervals to new medium. McClary (1940) did claim that, using a medium solidified with agar and containing 5 per cent dextrose, he had succeeded in culturing maize roots through 8 subcultures. He reported that all roots increased in length through a period of 115 days at an average rate of approximately 8 mm. per day.

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Attempts to repeat this work by Bonner (Bonner and Bonner 1948) and Robbins (1951) have however been unsuccessful, and it must be concluded that McClary's success was either due to the particular strain of maize used or to some unknown factor in his technique which has not been duplicated in later work. Bonner and Bonner (1948) in reviewing this subject concluded that »The roots of the *Gramineae* form a group which have resisted indefinite culture on any medium».

It is clear from many accounts that excised cereal-root tips, when transferred to culture media containing sugar, inorganic salts and B vitamins, often grow quite rapidly during the period immediately following excision from the seedling. This strongly supports the view that their subsequent failure is not a consequence of injury inflicted by excision, of lack of aeration, of the physical state of the medium, or of toxicity of the chemical substances initially present in the medium. Furthermore there is no evidence that changes in the medium are responsible for failure to grow on subculture for this has not been found to be affected by the frequency of subculture or the volume of medium used per culture. It is therefore suggested that the cessation of growth in culture is due either (1) to a requirement for some growth factor(s) or metabolite(s) not supplied or supplied under unsuitable conditions by the root-culture media hitherto used or (2) to a derangement of root metabolism resulting from detachment from the shoot and causing the accumulation of lethal substances in the root — this implies some influence of the shoot which can never be supplied by the culture medium, for otherwise it becomes identical with (1) above.

In a renewed approach to the problem of culturing excised cereal roots it seemed desirable to work with a species or strain in which the initial growth was fairly high, in which the decline in growth did not prevent at least one successful subculture, and in which lateral formation in culture would make possible exploration of the possibility of establishing clones by sector cultures. From a preliminary survey carried out in our laboratory a sample of Petkus rye was chosen. This grew well, persisted in certain media for at least 4 passages, and showed a marked growth stimulation in response to additions of a mixture of a yeast extract, peptone and casein hydrolysate.

This choice was also supported by the report by Lowe (1950) that he had studied carbohydrate utilisation of excised rye roots growing in Burström's medium (Burström 1941) and had not encountered the rapid decrease in meristem size recorded by Almqvist (1949) in his work with other excised grass roots.

The present paper describes the successful subculture of excised rye roots through many passages, the maintenance and building up of a clone of roots and the partial identification of the nutrient requirement of this clone. The

demonstration that excised rye roots can be obtained in continuous culture by a simple addition to White's medium may indicate the nature of the general problem posed by the resistance of cereal roots to culture. It should however be emphasised that the technique and media used do not make possible the culture of other cereal roots or even of roots from a high proportion of the grains in our rye sample.

Experimental

General culture technique

Seeds of Petkus II rye (Linton Pure Seed Co, Cambridge) were sterilised by shaking in 1 per cent of the detergent Lissapol for one minute, followed by 0.1 per cent aqueous mercuric chloride for 20 minutes. The mercuric chloride was removed by five washes with sterile double-distilled water and the seeds plated out in sterile petri-dishes containing a circle of Whatman No. 1 filter paper moistened with double-distilled water. After 3 days germination at 25° C, 12 mm. root tips were excised from seminal roots and transferred to the culture medium.

The culture media used were the standard culture medium employed for the culture of excised tomato roots (Street and McGregor 1952) and modifications of this. 125 ml. narrow-necked Pyrex Erlenmeyer flasks containing 50 ml. of culture medium were inoculated with single root tips or sector pieces. Unless otherwise indicated, culture vessels and medium were sterilised by autoclaving at 15 lbs. for 5 minutes. Cultures were incubated at 25° C in incubators with glass doors thereby exposing the cultures to diffuse daylight.

Establishment and maintenance of a clone

Excised rye roots grew poorly in the standard culture medium. Addition to this medium of 10 mg./l. each of Difco Bacto yeast extract, B.D.H. peptone and Difco Bacto casein hydrolysate improved growth. The clone was initiated from a root growing in this medium: the root chosen had been initiated from a 12 mm. seedling root tip and when subcultured on the 18th day was 85 mm. long and had 10 primary laterals (total length of 75 mm) and 11 secondary laterals. This root was used to establish 2nd-passage sector cultures. From this point the clone was subcultured every 14 days. The technique used was as follows: - 12 ± 2 mm. root tips were excised from the primary laterals of the sectors and these tips were used to set up experiments; at the same time, from the primary laterals, sectors each approximately 15 mm. long and bearing 5 laterals 10—20 mm. long were excised and used to maintain the clone.

The medium supplemented by 10 mg./l. yeast extract, peptone and casein hydrolysate was used for 7 passages. Subsequently the clone was maintained in standard culture medium supplemented by 30 mg./l. of the yeast extract. In all, the clone was maintained without diminution in growth rate of the sector cultures through 38 passages each of 14 days.

General technique of experiments

The 12 mm. root tips excised from sectors were distributed evenly between treatments and incubated for 14 days. Each treatment was represented by 10 replicates. At the end of the growth period, calculations were made from measurements on the individual roots of average values for: increase in length of main axis (total length minus 12 mm.), number of primary laterals, total length of laterals, number of secondary laterals, and in certain experiments of the dry weight.

Evolution of a satisfactory culture medium.

Preliminary experiments showed that: Standard culture medium plus 30 mg./l. yeast extract was at least equal in growth-promoting activity to medium supplemented with 10 mg./l. each of yeast extract, peptone and casein hydrolysate. An ether-exhausted aqueous solution of the yeast extract was equal in growth-promoting activity to untreated yeast extract. Casein hydrolysate alone was much inferior to either yeast extract or peptone alone when tested over the range 20—100 mg./l. At equal concentrations peptone was slightly superior to yeast extract giving significantly higher values for increase in main-axis length and total lateral length. Omission of glycine (the only amino-acid in White's medium) was without significant effect on the growth in standard culture medium or in this medium supplemented with yeast extract. Sucrose and dextrose supported a similar level of growth in media containing 30 mg./l. yeast extract. When tested over the concentration range 0.25 to 8.0 per cent. optimum growth with sucrose was obtained at 4 per cent. optimum growth with dextrose at 2 per cent. The ability of sucrose to support growth did not depend on its partial inversion during autoclaving of the medium. Media of the same growth-promoting activity to autoclaved medium were obtained when the sucrose was autoclaved separately in aqueous solution or was incorporated by aseptic addition after previous treatment with dry ether.

The higher growth-promoting activity of the yeast extract and peptone and the fact that increased lateral growth was the most marked feature of the stimulation, suggested that amino-acids might be involved (Skinner and Street, 1954). The analysis of Difco yeast extract supplied by the manufacturers had previously proved valuable in identifying the amino-acids which stimulate the growth of excised groundsel roots (Skinner and Street 1954). In view of this it was decided to attempt the identification of the active constituent(s) of our yeast extract despite the slight superiority of our peptone sample in stimulating the growth of rye roots. A medium containing 1 per cent sucrose, with glycine omitted, and with 30 mg./l. yeast extract added was used as a basis for comparison with experimental treatments involving

additions of known amino-acid mixtures, of individual amino-acids and of related compounds. Glycine, which is normally included in standard culture medium was omitted in order to avoid antagonistic effects particularly when using single amino-acids or simple mixtures (Harris 1953). The yeast-containing medium specified above is subsequently referred to as the *yeast-control medium*. Standard culture medium containing 4 per cent sucrose, with glycine omitted and *without* the yeast extract addition is referred to as *control medium*.

Growth-promoting activity of the amino-acids of Difco Yeast Extract

Table 1 which is based on the analysis of Difco yeast extract, shows for each amino-acid the content in 30 mg. extract. It is arranged to indicate the amino-acids included in each of the three fractions tested singly and in all combinations for their ability to reproduce the growth-promoting activity of the yeast extract (and the total amino-acid mixture (Fractions 1+2+3).) The results of such an experiment are shown in Figure 1. It can be seen from this figure that although main-axis growth is stimulated by yeast extract its main effects are on lateral number and particularly on total lateral length. This latter measurement has proved the most consistent and sensitive criterion in assessing growth-promoting activity, and is used below to compare the relative growth-promoting activities of the different treatments. The combinations Fraction 1+2+3 or 2+3 both supported about 82 per cent of the growth obtained in the yeast-control medium. Fractions 1 and 2 either singly or in combination had no growth-promoting effect. Fraction 3 and

Table 1. *The amounts of individual amino-acids supplied by 30 mg. Difco Yeast Extract.*

Stock Solution	Amino-acids used	Amount. mg.
<i>Fraction 1.</i>	L-aspartic acid	1.36
	L-glutamic acid	1.39
	L-isoleucine	0.88
	L-leucine	1.08
	DL-valine	1.02
<i>Fraction 2.</i>	L-arginine	0.23
	L-histidine mono-HCl	0.28
	L-lysine di-HCl	1.20
	L-phenylalanine	0.66
	L-tyrosine	1.18
<i>Fraction 3.</i>	DL-methionine	2.38
	DL-threonine	1.02
	L-tryptophane	0.26

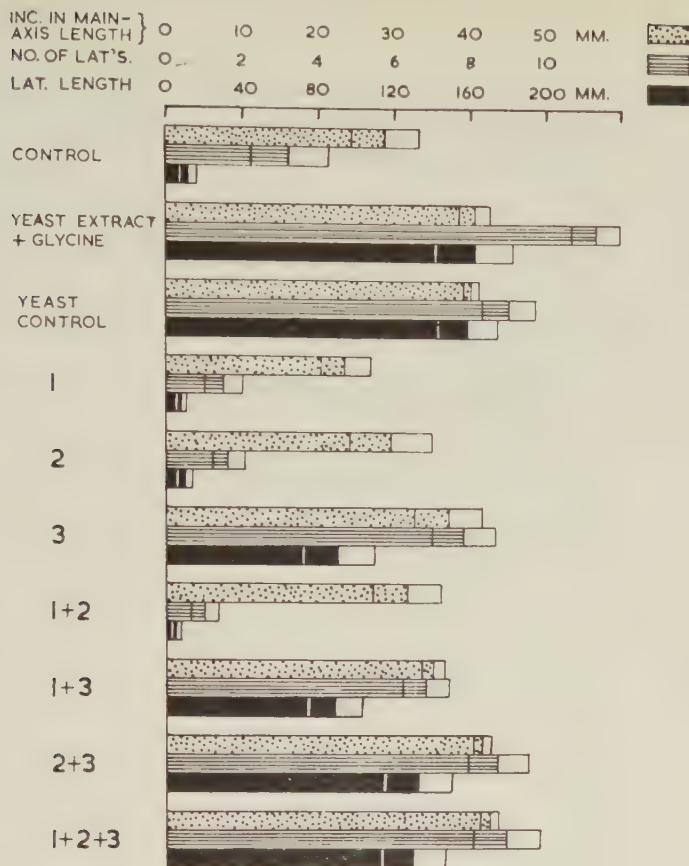


Figure 1. Effects on the growth of excised rye roots of 3 fractions of the amino acids present in 'Difco' yeast extract (the composition of these fractions is shown in Table 1) when added singly (1, 2 and 3) and in combinations to control medium. Each histogram represents the mean values for 10 replicates; in each case \pm the standard error is represented by vertical lines on either side of the mean value.

the combination Fraction 1 + 3 were clearly stimulatory, though the level of growth was below that achieved in the combinations 2 + 3 and 1 + 2 + 3. Fraction 3 alone had approximately 50 per cent of the growth-promoting activity of yeast extract. Fraction 2, though lacking in activity, enhanced the activity of Fraction 3. The eight amino-acids contained in the combination Fraction 2 + 3 were then tested singly (Figure 2). The mixture of all eight amino-acids (Fraction 2 + 3) or L-tryptophane alone had the same growth-promoting activity; in the present experiment this was approximately 65 per cent of that of yeast extract. The other amino-acids tested individually

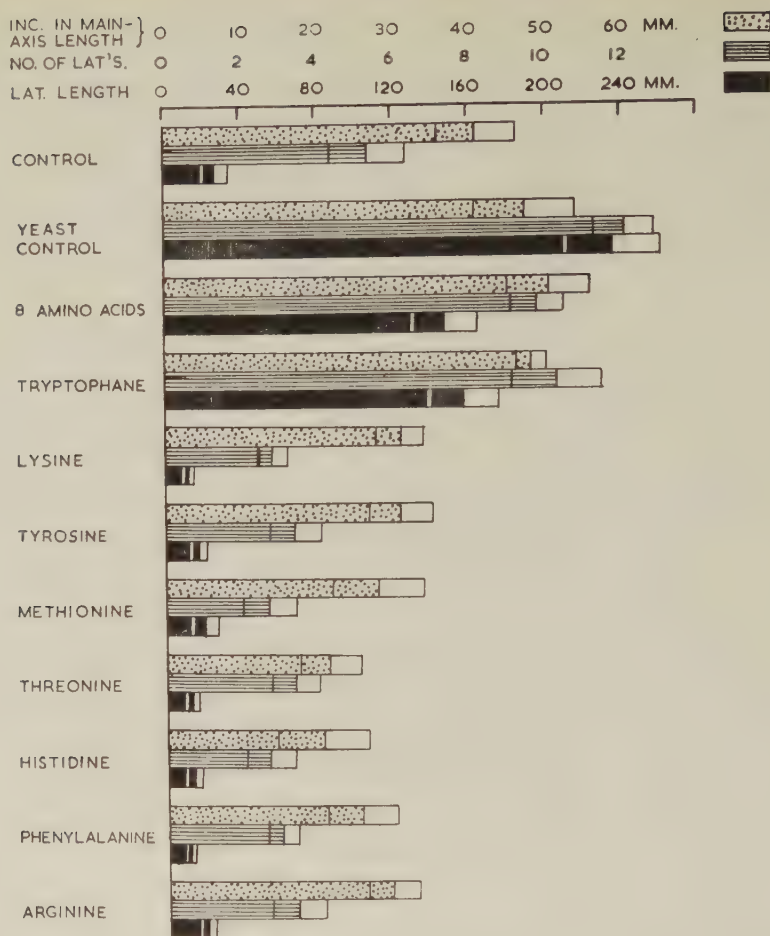


Figure 2. Effects on the growth of excised rye roots of adding singly to control medium each of the 8 amino acids present in Fractions 2+3. Growth in yeast control medium and in control medium+Fractions 2+3 (8 amino acids) shown for comparison. Explanation of histograms as in Figure 1.

either had no growth-promoting activity or were inhibitory. L-tryptophane appeared therefore to be responsible for the growth-promoting activity of the combination Fraction 2+3. The superiority of this combination over Fraction 3 alone was possibly due to antagonism of threonine inhibition by an amino-acid of Fraction 2. The conclusion that the growth promoting activity of Fraction 2+3 was due to tryptophane was confirmed by an experiment in which each amino-acid was in turn omitted from this combination (Figure 3). The seven treatments containing L-tryptophane stimu-

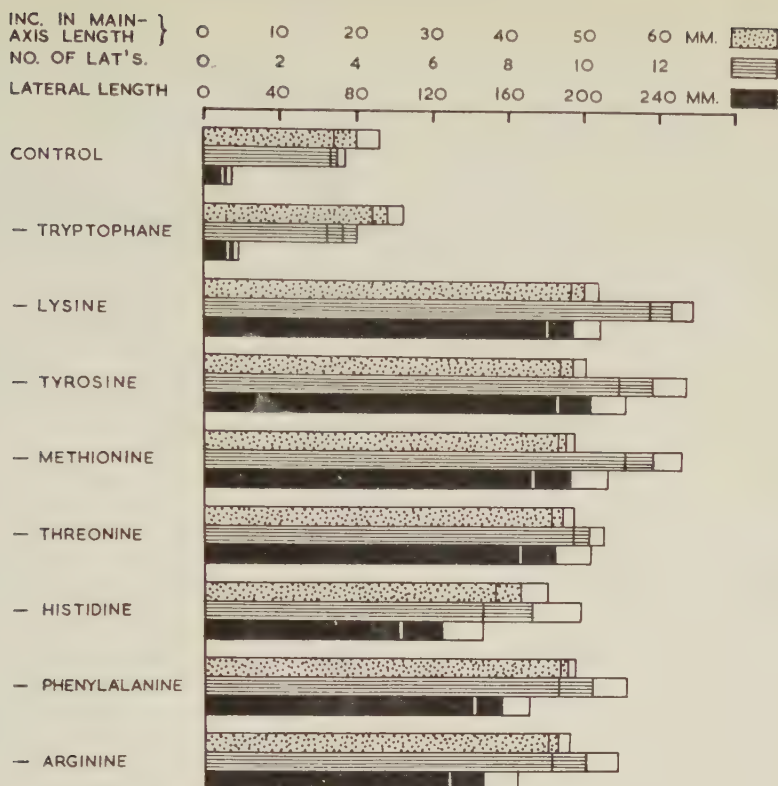


Figure 3. Effects on the growth of excised rye roots of additions to control medium of 7 of the 8 amino acids present in Fractions 2+3; a different amino acid was omitted in each treatment and the omitted amino-acid is indicated against each histogram set.

Explanation of histograms as in Figure 1.

lated growth, while the treatment with L-tryptophane omitted supported the same level of growth as control medium.

The fact that L-tryptophane alone was equal in growth-promoting activity to the combination Fraction 2+3 (Figure 2) and this in turn to the combination Fraction 1+2+3 (Figure 1) showed that L-tryptophane reproduced the stimulation of yeast extract which arises from its known amino-acid content (approximately 80 per cent of the total activity).

Subsequent experiments not now reported in detail showed that biotin, riboflavin and yeast nucleic acid, over a wide range of concentrations, were without growth-promoting activity to excised rye roots. The nature of the constituent(s) in yeast extract which accounts for its superiority over L-tryptophane has not been further investigated.

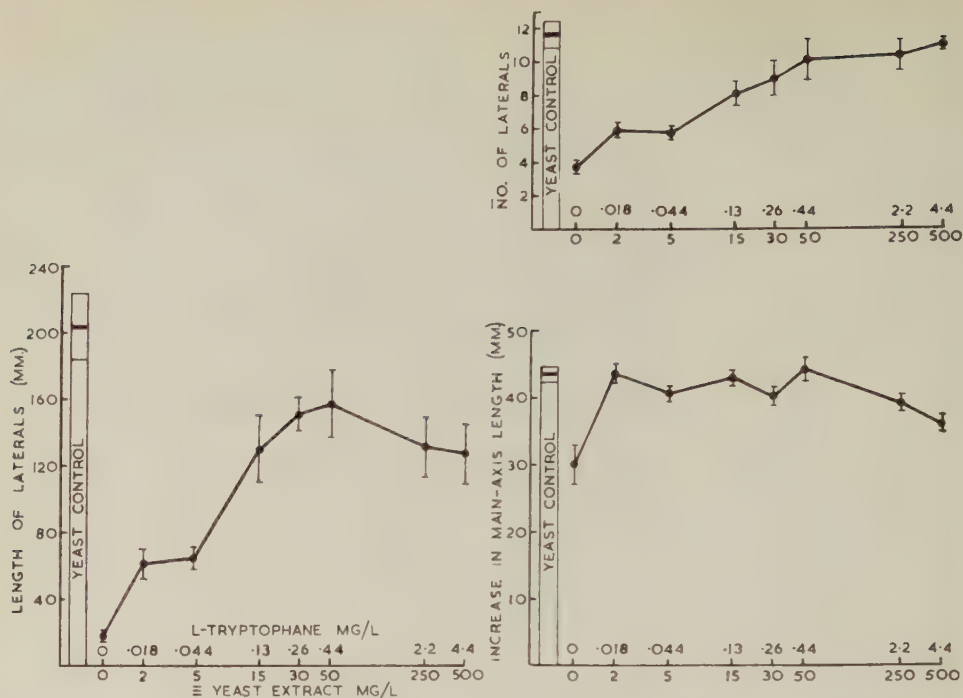


Figure 4. Effects on the growth of excised rye roots of different concentrations of L-tryptophane added to control medium compared with growth in 'yeast control' medium. Each point represents 10 replicates and the vertical line at each point represents $2\times$ the standard error. The concentration scale is logarithmic.

The effect of L-tryptophane concentration was tested over the range 0.018—4.4 mg./l. (equivalent to the reported tryptophane content of 2—500 mg./l. of the yeast extract). The results (Figure 4) show that maximum growth stimulation occurred at 0.44 mg./l. (the equivalent of 50 mg./l. yeast extract

the minimum yeast extract concentration at which full growth-stimulation is obtained). Marked growth-stimulation was recorded at the lowest tryptophane concentration tested.

Effects of yeast extract and of L-tryptophane on the growth of roots repeatedly subcultured by excision of main-axis tips

The yeast extract was clearly essential for the satisfactory growth of the sector cultures of the rye clone. However, it did not markedly stimulate the main-axis growth of tip cultures grown for a single 14-day passage.

Experiments were therefore undertaken to see how far yeast extract and

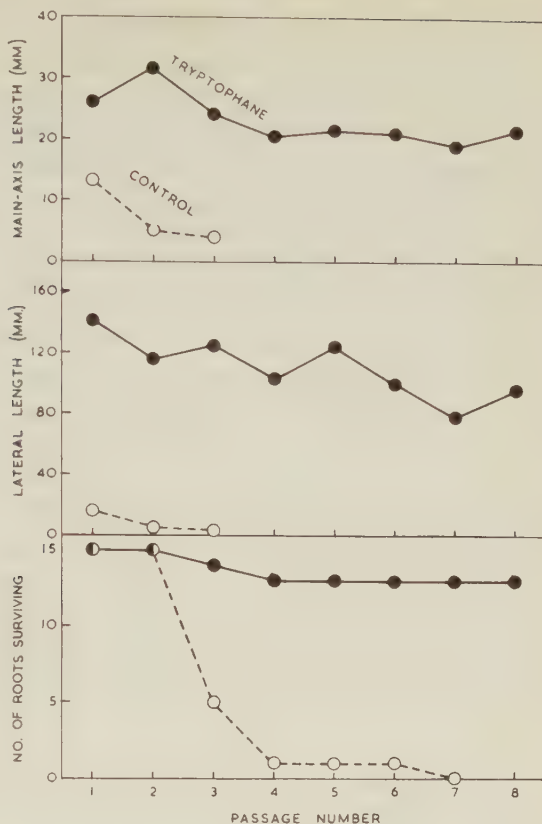


Figure 5. Growth and survival of excised rye roots subcultured by repeated excision of main axis tips in control medium and control medium supplemented with 0.26 mg./l. L-tryptophane (the tryptophane concentration supplied by 30 mg./l. yeast extract).

tryptophane would improve the persistence and level of growth of individual main-axis meristems repeatedly subcultured (technique described by Street, McGonagle, and Roberts 1953). Survival of activity in main-axis tips was found to be high in culture medium containing either yeast extract or tryptophane: the few root tips which failed to grow on subculture were brown and swollen. By contrast when cultured in standard medium less than half the roots survived beyond the second passage and usually all roots ceased growth by Passage 4. Root tips ceasing growth in this medium were white and very thin and tapering. The early and rapid decline in survival observed in standard medium was associated with a fall in growth to a very low level. The growth of roots cultured in yeast extract or tryptophane-supplemented medium fluctuated from passage to passage, but there was no evidence of any overall decline in growth rate with time. The results of an experiment comparing growth and survival in standard medium and in this medium supplemented by addition of 0.26 mg./l. L-tryptophane (the trypto-

phane level supplied by 30 mg./l. yeast extract) are shown in Figure 5. The addition of yeast extract or L-tryptophane therefore makes possible the continued growth of the apical-tip meristems of our Petkus-rye clone. The few tips which did cease activity in these media resembled tomato root tips showing the 'ageing' phenomenon (Street, McGonagle, and Roberts 1953). This 'aging' was ascribed to the accumulation of a critical supra-optimal level of a root hormone (Street 1954 a). By contrast the cessation of meristematic activity in standard medium suggests rather the depletion from the apices of a growth-promoting substance which the rye roots are either unable to synthesise or cannot synthesise at the critical rate required for the maintenance of meristematic activity. Tomato root tips inhibited by an excess of the anti-auxin, NMSP, are similarly thin, white and floating.

Nature of the growth-stimulation caused by addition of L-tryptophane to standard medium

(a) *Effects on growth of substances related to tryptophane*

In the present investigation, this problem has been approached by substitution experiments in which a search has been made for compounds related to tryptophane which would cause a similar stimulation of growth.

(i) *Nicotinic acid and L-Kynurenine.* — Work with a variety of organisms supports the view that L-tryptophane acts as a precursor in nicotinic acid synthesis by way of L-kynurenine (Mitchell 1950). Recently several workers (Gustafsson 1949, Galston 1949, Wiltshire 1953) using higher plants, have obtained results pointing to the operation of this metabolic pathway. Nicotinic acid however, is present in standard culture medium at a concentration of 0.5 mg./l. and it therefore seemed unlikely that the tryptophane stimulation arose from its combating a nicotinic acid deficiency in our roots. Experiment showed that omission of nicotinic acid from standard medium, or from media containing either 0.44 or 2.2 mg./l. L-tryptophane slightly enhanced growth, and that increase in nicotinic acid concentration up to 5.0 mg./l. progressively retarded growth of both the main axis and laterals. L-kynurenine at 0.45 mg./l. (equivalent in molarity to 0.44 mg./l. L-tryptophane) was without significant effect on excised rye-root growth. The tryptophane stimulation therefore does not seem to be explicable on the basis of a metabolic relationship between tryptophane and nicotinic acid.

(ii) *Indole and Serine.* Tatum and Bonner (1944), using *Neurospora* mutants, first obtained evidence of tryptophane synthesis by condensation

of indole and serine; the immediate precursor of indole being anthranilic acid. Subsequent work with other fungi and with bacteria has shown the wide occurrence of this pathway of synthesis. Growth of excised rye roots was therefore compared in media supplemented by anthranilic acid, indole, DL-serine and L-tryptophane added singly and in various combinations. L-tryptophane was used at 0.44 mg./l., anthranilic acid and indole at the same molarity and DL-serine at twice this molarity (on the assumption that only L-serine would be involved in L-tryptophane synthesis). There was no evidence of serine toxicity at the concentrations used in this and the succeeding experiment: tryptophane stimulation was not depressed by serine, nor did serine depress growth as compared with the control medium. Serine, indole and anthranilic acid added separately and also combinations of serine and anthranilic acid all failed to stimulate growth. Serine plus indole caused a significant ($P=0.05$) stimulation of lateral growth (equal to 20–30 per cent of the full tryptophane stimulation). This stimulation of lateral growth by serine plus indole was confirmed in a second experiment but could not be significantly enhanced by alterations in the serine: indole ratio (over the range from 0.5 : 1 to 64 : 1). These results suggest that excised rye roots supplied with serine plus indole can effect a slow synthesis of tryptophane or some related compound and that this accounts for their small but reproducible stimulating effect.

(iii) *Gramine, Skatole and DL-Tryptophanol*. — Gramine, known to be synthesised from tryptophane in barley shoots (Bowden and Marion 1951), when tested over a concentration range equivalent in molarity to 0.13 to 1.32 mg./l. L-tryptophane, was inactive.

Skatole, a decomposition product of tryptophane in animal cells and a substance active in the oat-coleoptile curvature test (Glover, 1936), was also inactive over a concentration range equivalent in molarity to 0.13 to 4.4 mg./l. L-tryptophane.

DL-tryptophanol, either incorporated into the medium before autoclaving or sterilised separately by filtration (Street, McGonagle, and Lowe 1951), stimulated both main-axis and lateral growth. If added in sufficient quantity it fully reproduced the growth stimulation resulting from addition of L-tryptophane. The lowest concentration of tryptophanol giving the full stimulation varied from experiment to experiment but did not fall below $4\times$ the tryptophane molarity having equal activity. Using chromatographic methods, it was subsequently shown (p. 255) that the tryptophanol sample contained a substance having the same R_f as IAA. It now seems probable that the activity of tryptophanol is to be explained in terms of this contaminant.

(iv) *β -Indolylacetic acid (IAA)*, *β -Indolylacetonitrile (IAN)* and *D-Tryptophane*. — Bonner (1932) showed that *Rhizopus suinis* developed high auxin activity on a substrate containing peptone, and Thimann (1935) showed that this was due to oxidation by the fungus of tryptophane present in the peptone. Subsequently a wide variety of plant tissues have been shown to have the ability to produce auxin when supplied with tryptophane (Larsen 1951, Audus 1953). In these experiments definite identification of the auxin produced has not been carried out although it has generally been assumed, in the absence of evidence to the contrary, that it was IAA.

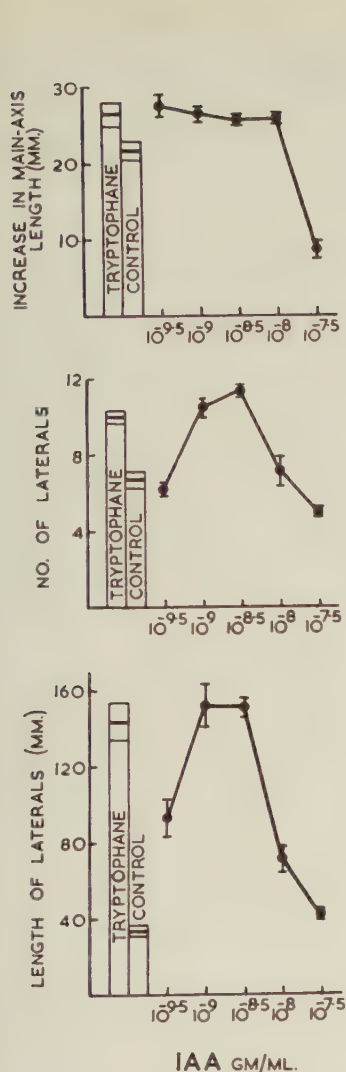
Concentrations of IAA below 10^{-10} g./ml. have little effect on the growth of excised rye roots. Inhibition of growth occurs at and above a concentration of $10^{-7.5}$ g./ml. Marked growth stimulation, particularly of lateral growth, occurs within the range $10^{-9.5}$ — 10^{-8} g./ml. The growth stimulation at 10^{-9} and $10^{-8.5}$ g./ml. IAA is equal to that obtained with 0.44 mg./l. L-tryptophane (Figure 6).

A similar growth stimulation occurred with IAN. Maximum lateral stimulation occurred at 10^{-9} g./ml. (Figure 7). 10^{-10} g./ml. was clearly stimulatory and 10^{-8} g./ml. was inhibitory. The growth response to IAA and IAN was of the same order as that obtained with L-tryptophane. These substances were most effective in stimulating growth at approximately 1/400th of the optimum molar concentration of tryptophane. The same order of concentration of either IAA and IAN is required for optimum stimulation; inhibition becomes evident at a slightly lower concentration with IAN than with IAA. The response of excised rye roots to IAA and IAN contrasts with that of the seedling cress root (Bentley and Bickle 1952) and the excised tomato root (Street, McGregor, and Sussex 1954) where, using root inhibition as the criterion of activity, IAN was found to be about $100\times$ less active than IAA.

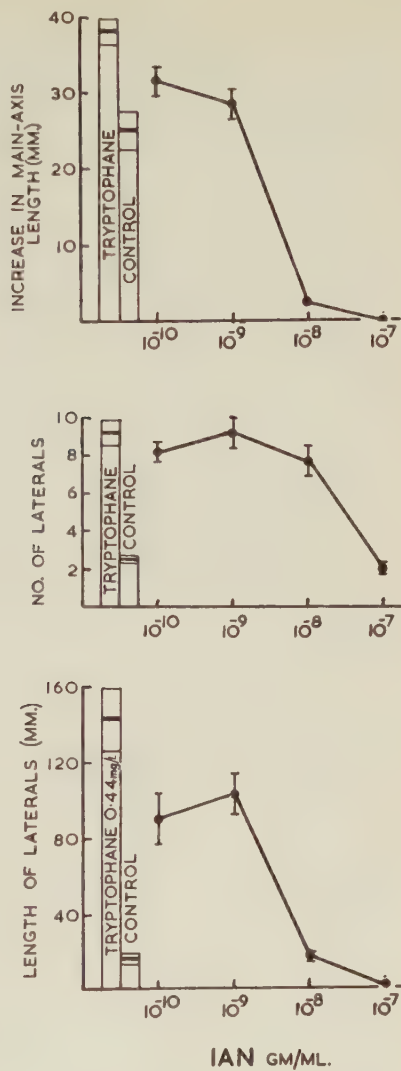
The ability of the optically inactive substances IAA and IAN to reproduce the L-tryptophane stimulation led to an examination of the activity of D-tryptophane. This revealed that D-tryptophane was exactly equivalent in activity to L-tryptophane in its effect on main axis and lateral growth.

(b) *The activation of tryptophane on heating in aqueous solution*

Gordon and Wildman (1943) showed that when tryptophane was heated in dilute alkaline solution it was converted into a substance (presumed to be IAA) active in the *Avena* coleoptile curvature test. When a solution of tryptophane in distilled water was boiled for 7 hours it also yielded detectable quantities of auxin. Kulescha and Gautheret (1949) have also reported the formation of a substance active in the *Avena* test when tryptophane solutions are autoclaved. Since in the experiments described in the earlier sections of the present paper, tryptophane containing media had been sterilised by



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Figure 6. Effects on the growth of excised rye roots of different concentrations of β -indolyl-acetic acid added to control medium compared with L-tryptophane added at 0.44 mg./l. (the optimum concentration). Replication and representation of standard errors as in Figure 4.

Figure 7. Effects on the growth of excised rye roots of different concentrations of β -indolyl-acetonitrile added to control medium compared with L-tryptophane added at 0.44 mg./l. Replication and representation of standard errors as in Figure 4.

autoclaving it became important to examine how far their growth promoting activity was due to formation during sterilisation, of IAA from tryptophane. The full growth-promoting activity of the tryptophane addition could be accounted for on this basis if the concentration of IAA established was of the order of 1/400th the molarity of the tryptophane addition.

(i) *Demonstration of the activation of tryptophane by heating in aqueous solution.* — The growth promoting activity of autoclaved tryptophane and of 'aseptically added' tryptophane has been compared. The technique for the aseptic addition was as follows: crystals of L-tryptophane were weighed using sterile forceps, spatula and watchglasses and then dissolved in sterile double-distilled water. This solution was then added to culture flasks containing an appropriately concentrated autoclave-sterilised standard medium. The volume and concentration of the medium was such that it was adjusted to the correct strength and volume (50 ml.) by the appropriate volume (varying according to the concentration of tryptophane being added) of the aseptically prepared tryptophane solution. These manipulations were carried out in a sterile transfer room (Street 1954b). The results are shown in Figure 8.

The 'aseptically added' tryptophane was of very low activity compared with the autoclaved tryptophane. A subsequent experiment demonstrated that 'aseptically added' tryptophane did not decrease the growth occurring in presence of autoclaved tryptophane and thereby eliminated the possibility that unheated tryptophane contains an inhibitory substance rendering it inactive and that the activation on autoclaving is due to destruction of an inhibitor. These results suggested that the growth stimulation resulting from addition of tryptophane to the medium was due to the formation from tryptophane of an auxin during autoclaving.

It was considered that separation and ultimate identification of the postulated auxin in heat-activated tryptophane would be facilitated if activation could be obtained by controlled heating in aqueous solution rather than by autoclaving in the presence of the constituents of the culture medium. A solution of L-tryptophane (22 mg./l.) in double-distilled water was therefore refluxed for 25 hours, samples being withdrawn aseptically after 1, 5 and 25 hours. These samples and aseptic dilutions prepared from them were used to achieve concentrations in sterile medium of 0.017, 0.086 and 0.43 mg./l. L. tryptophane. When tested for their stimulatory effect on excised rye roots it was shown that activation equivalent to that resulting from autoclaving with the medium occurred after 5 hours of refluxing in aqueous solution and that extension of the period of heating beyond this slightly decreased activity.

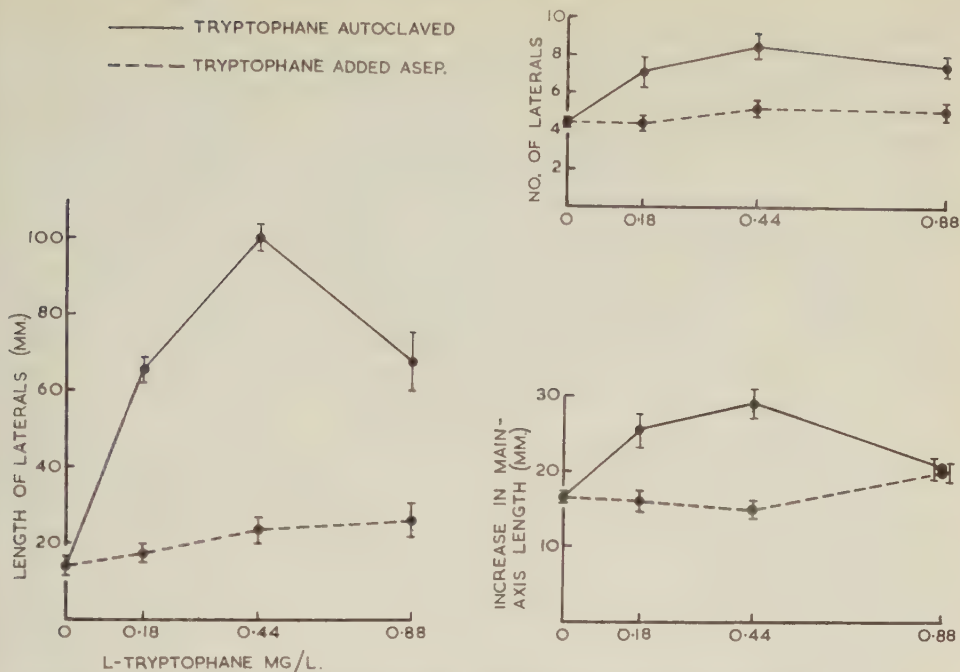


Figure 8. Effects on the growth of excised rye roots of various concentrations of L-tryptophane 'added aseptically' to previously autoclaved control medium compared with similar concentrations of L-tryptophane autoclaved with the control medium. Replication and representation of standard errors as in Figure 4.

(ii) *Partition of activity between ether and water.* — The partition of L-tryptophane, IAA and IAN between ether and water was studied by chromatography.

Chromatograms were run on Whatman No. 3 paper strips for 12 hours using as solvent isopropanol (80 parts by volume) — 0.15 N ammonia (20 parts by volume). The chromatograms were then dried in a current of warm air (approx 45° C) and developed by spraying with 1 per cent p-dimethylaminobenzaldehyde in approximately 1-N hydrochloric acid (Block, Le Strange, and Zweig, 1952). The initial colours resulting were: purplish-pink with tryptophane and tryptophanol blue with IAA, yellow with IAN. The Rf values obtained under these conditions were tryptophane main spot 0.2–0.24, subsidiary spot 0.3–0.35, IAA 0.4–0.5, tryptophanol 0.75–0.80, IAN 0.9–0.95. Our tryptophane sample gave a single spot on Whatman No. 1 paper but with the No. 3 paper, chosen to achieve heavier loading and using isopropanolammonia there was below the main spot a paler spot which did not separate from the main one and which was centred around Rf .3–.35.

To test the extractability of IAA and IAN from standard medium by ether the following experiment was carried out. 50 ml of standard culture medium

(pH 4.85) containing tryptophane, IAA (0.1 mg.) and IAN (0.1 mg.) was shaken with an equal volume of anaesthetic ether. Evaporation of the ether extract to dryness and solution of the residue in water followed by chromatography showed, after development, dense spots of both IAA and IAN. A second ether extract after adjustment to pH 3.0 was almost completely negative, there being only the faintest indication of colour at the IAA position. This result indicated that, at pH 4.85, the partition of IAA and IAN as between culture medium and ether is overwhelmingly in favour of solubility in the ether. The growth promoting activity of autoclaved tryptophane medium should, if activation is due to formation of either of these auxins, pass almost completely into the ether when equal volumes of ether and medium are shaken together.

Standard culture medium containing 0.44 mg. L-tryptophane per 20 ml. was autoclaved (pH after autoclaving 4.5). Some of this was added aseptically to sterile control medium to give tryptophane-autoclaved medium (0.44 mg./l). 20 ml. was extracted in a sterile separating funnel with 20 ml. anaesthetic ether. The medium fraction was freed from ether by aeration for 3 hours at 40° C under aseptic conditions and then added 1 ml per flask to flasks containing 49 ml. of control media ('tryptophane water fraction'). The ether fraction was evaporated to dryness at 40° C under aseptic conditions and the residue was dissolved in 100 ml. sterile double-distilled water; this was added, 5 ml. per flask, to flasks containing 45 ml. of a solution containing the constituents normally present in 50 ml. culture medium. This treatment is referred to as the 'tryptophane ether fraction'.

Both the 'tryptophane water fraction' and the 'tryptophane ether fraction' had high activity; the activity of the 'water fraction' was quite equal to that of the total tryptophane (Figure 9). The partitioning of activity between medium and ether did not correspond to that which would occur if activation is due entirely to IAA and/or IAN formation. Either formation of these auxins during activation does not occur or if the activity of the 'ether fraction' is due to them then in addition there must be formed substance(s) preferentially soluble in the medium.

(iii) *Chromatographic examination of tryptophane for the presence of indole compounds arising during activation.*

A solution of L-tryptophane in double-distilled water was divided into three 800 ml. portions each containing 40 mg. tryptophane. The first portion was left unaltered, to the second portion 0.01 mg. IAA was added, and the third portion was refluxed for 5 hours. Each solution was then separately adjusted to pH 3.0 with hydrochloric acid and extracted 4 times with 100 ml. anaesthetic ether. The combined ether extracts of each solution were then evaporated to small volume in vacuum at 40° C and applied separately at the starting line of the chromatogram. After chromatography and spraying with p-dimethylaminobenzaldehyde the ether extracts of the untreated and refluxed solutions showed only a single spot of Rf 0.21 (corre-

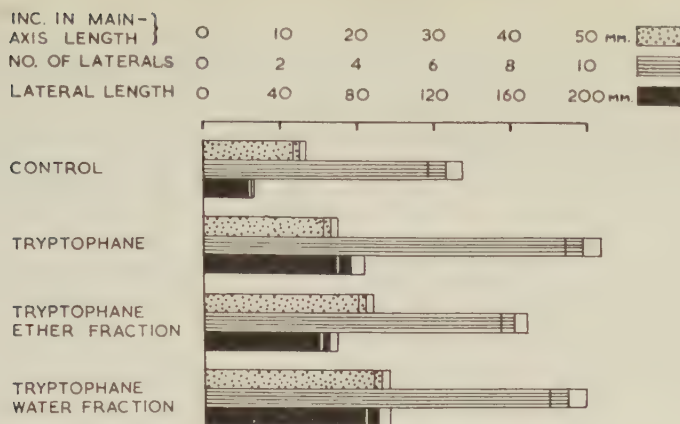


Figure 9. Partition of the growth promoting activity of 'activated' tryptophane between ether (tryptophane ether fraction) and medium (tryptophane water fraction). Activity tested by comparison of growth in control medium with that in medium to which tryptophane or the ether or water fraction had been added. Explanation of histograms as Figure 1.

sponding to tryptophane). The ether extract of the solution to which had been added IAA to the extent of 0.025 per cent of its tryptophane content showed in addition to the tryptophane spot, a second clearly defined spot of R_f 0.42 (corresponding to IAA). This experiment was performed on three occasions with the same result.

Assessed by its stimulating effect on excised rye root growth IAA is equivalent to about $400\times$ its own weight of activated tryptophane. If the activity of heated tryptophane solutions depends upon their IAA content then this should be about 0.25 per cent of their initial tryptophane content. The chromatographic procedure described above easily detected IAA present in a solution to the extent of as little as 0.025 per cent of its tryptophane content. It failed, however, to reveal in the refluxed solution of the same tryptophane content any indication of IAA or other substance giving a colour with p-dimethylaminobenzaldehyde and differing in R_f from tryptophane. If IAA is formed during the heat activation of tryptophane this must take place only to a very small extent and could only account for a small fraction of the total activity.

(iv) *Chromatographic examination of tryptophanol for the presence of indole compounds.*

320 mg. of DL-tryptophanol was dissolved in 2 ml. ethanol. This was added to 100 ml. distilled water when most of the tryptophanol came out of solution as a

fine precipitate. The solution was centrifuged and the precipitate filtered out. The filtrate was divided into two equal portions. 0.02 mg. IAA was added to one of these and then both solutions were adjusted to pH 3.0. Each solution was extracted 4 times with 25 ml. anaesthetic ether. In the same way as was described in section (iii) the combined ether extracts from each solution were evaporated and the residues applied separately to the starting line of a chromatogram. 0.02 mg. IAA was applied to a third spot on the starting line. The chromatogram was run and developed as before.

The results showed the presence of a substance in the tryptophanol sample which ran to exactly the same position as the IAA indicator spots of the other two chromatograms. If this substance is calculated as IAA, at least 1 part in 8,000 is present in the tryptophanol sample. Allowing that tryptophanol is required at 4 times the concentration of 'activated' tryptophane, and that 'activated' tryptophane is required at 400 times the concentration of IAA for similar levels of growth stimulation, this means that at the very least one-fifth of the optimal quantity of IAA is present in the concentration of tryptophanol which gives maximum stimulation. It therefore is possible that the growth stimulation caused by the addition of tryptophanol is due to contamination with IAA.

This demonstration that the tryptophanol is contaminated with a substance of the same *R_f* as IAA enhances our confidence that the chromatographic technique used would have revealed the presence of IAA in 'activated' tryptophane if it had been present there in significant amount.

Discussion

Excised roots of different species differ in their response to externally applied IAA. Excised tomato roots are unaffected by low concentrations and inhibited by higher concentrations (Street, McGregor, and Sussex, 1954). By contrast IAA at appropriate concentrations has been found to stimulate the growth of excised roots of pine (Slankis 1951), white lupin (Duhamet 1939), pea (Bonner and Koepli 1939, Naylor and Rappaport 1950), maize (Fiedler 1936, Kandler and Viereggs 1953) and wheat (Burström 1942). Excised rye roots represent an extreme example of growth stimulation by IAA; an effect which can also be produced by IAN.

The ability of IAA and IAN to reproduce the tryptophane stimulation of excised rye roots suggests that we are here concerned with a growth hormone requirement. This requirement is absolute in the sense that it must be met if the roots are to be maintained in continuous culture. The only similar case seems to be that reported by Naylor and Rappaport (1950) who succeeded in growing, for the first time, excised roots of pea var. World's Record

by adding to the basic medium cysteine plus tryptophane followed by autoclave sterilisation. Apparently addition of tryptophane alone was not tried. In contrast to work with excised roots, IAA has been found to be essential for the establishment and maintenance of callus cultures derived from a number of tissues. In one such case, that of a callus derived from Jerusalem Artichoke (Kulescha and Gautheret 1949, Kulescha 1949), it was shown that aseptically added DL-tryptophane used at 10–100 \times the effective IAA concentration had a similar, if smaller, stimulating effect. The callus tissue did not produce auxin (material active in the *Avena* test) in the absence of tryptophane but did so in its presence. These observations together with the experimental results now presented suggest the hypothesis that autoclaved tryptophane stimulates and makes possible the persistence of excised rye root growth by virtue of its content of growth hormone(s) and that the roots cannot actively transform tryptophane itself into the active material.

Excised roots, according to their behaviour towards externally applied auxins, can be separated into three groups:

(i) Roots which, during culture, accumulate an essential growth hormone to a supra-optimal concentration and are therefore either unaffected or inhibited by external auxin, e.g. tomato. Street (1954a), from anti-auxin experiments, has obtained evidence for the accumulation of a hormone (not IAA) to a supra-optimal concentration during the growth of excised tomato roots. There is evidence for a similar change in hormone concentration during the growth of pea seedling roots (Pilet 1951).

(ii) Roots in which the hormone concentration remains sub-optimal and which are therefore stimulated by external auxin, e.g. pea, maize, wheat. In the case of maize and wheat, attempts at subculturing these roots over a period of time shows that lack of growth hormone is not the primary factor preventing their continuous culture. Excised roots of maize and pea continue to produce auxin but the concentration present is clearly smaller than when they are attached to the plant (Nagao 1937, 1938; van Overbeek 1939).

(iii) Roots which have so low a content of hormone that an external auxin supply is essential for their continuous culture, e.g. rye.

Those roots which are stimulated by an external supply of auxin do not seem to show marked specificity for a particular substance. Würgler (1942) showed that α -naphthaleneacetic acid, β -indolylpropionic acid, IAA, *cis*-cinnamic acid or phenylacetic acid were all capable, to varying extents, of stimulating the growth of excised maize roots. Kandler (1953) found that the stimulation of excised maize roots by 2, 4-dichlorophenoxyacetic acid and α -naphthaleneacetic acid was of the same order as that obtained by IAA. The present work shows excised rye roots to be equally stimulated by 'activated' tryptophane, IAA and IAN. Street (1954a, 1955) found that

IAA and α -naphthaleneacetic acid both enhanced the natural 'ageing' occurring in excised tomato roots during repeated subculture.

The response to the anti-auxins, α -(1-naphthylmethylsulphide)-propionic acid and 1-naphthoxyacetic acid, of excised tomato roots depressed in growth by natural accumulation of hormone during repeated subculture is, however, quite different from that of roots inhibited by IAA. (Street 1954a, 1955). Clearly studies of the growth effect of externally applied synthetic auxins do not permit conclusions to be drawn regarding the identity of the natural hormones. However, it is mainly from work of this latter kind that an important role in the control of root growth has been ascribed to IAA. Van Overbeek and Bonner (1938) showed that the auxin extractable from excised pea roots was easily destroyed by alkali and therefore was probably not IAA. More recently, indications that roots contain several distinct growth hormones has come from chromatographic studies of ether extracts of seedling roots of pea, bean and maize (Bennet-Clark and Kefford 1953). In addition to providing evidence of the occurrence of IAA and possibly also of IAN, two other growth substances were detected (inhibitor β and accelerator α).

Tryptophane may be the precursor of one or more root hormones but its transformation may proceed extremely slowly or not at all in the excised rye root. The activity of indole plus serine, however, raises the possibility that the inactivity of 'aseptically added' tryptophane may result from a very slow rate of entry. Interest in the heat-activation of tryptophane arises from the evidence that it is not due primarily to IAA formation. This conclusion is supported by Avery and Berger (1943) who have pointed to the instability of the *Avena*-test activity of heated tryptophane solutions to strong alkali. Clearly, understanding of the problems raised by our results waits upon further knowledge of the natural root hormones and of the chemical change(s) involved in tryptophane activation.

The addition of yeast extract or tryptophane has made possible the continuous culture of a clone of excised rye roots. Other experiments (unpublished) show, however, that these media and the culture technique described only make possible the building up of clones from about 10 per cent of the grains of our rye sample, and fail to establish clones of other cereal roots tested. The present work, therefore, does not solve the problem of the culturability of excised monocotyledonous roots in general, although it may indicate that the essential condition for success is supplementation of the culture medium with growth factors (of which one may be that exposed in our present work with rye roots).

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Summary

1. The growth of excised roots of Petkus 11 rye in modified White's medium is stimulated by addition of B.D.H. Peptone or Difco Yeast Extract; the most marked stimulation is in lateral growth. Eighty per cent of the growth stimulation by the yeast extract arises from its content of L-tryptophane.
2. A clone of excised rye roots was established as sector cultures and maintained without diminution in growth rate through 38 passages of 14 days duration in medium supplement with 30 mg./l. Difco Yeast Extract.
3. The addition of yeast extract or L-tryptophane make possible the continued growth through successive passages of the main axis meristems of the clonal roots.
4. Nicotinic acid, L-Kynurenine, gramine and skatole did not stimulate growth. IAA and IAN at 1/400th the optimum molarity of tryptophane were equal to it in growth-promoting activity. D- and L-tryptophane had the same activity.
5. Tryptophane added aseptically to previously autoclaved medium does not stimulate growth. The tryptophane is 'activated' during autoclaving or when refluxed in distilled water.
6. When medium containing 'activated' tryptophane is shaken with ether, the partition of activity between ether and medium does not correspond with that which would be expected if activation is due to formation of IAA and/or IAN from the tryptophane during heating.

Examination of 'activated' tryptophane by chromatography also supports the view that IAA formation does not account for its activity.

7. The culture technique described only makes possible the establishment of clones from about 10 per cent of the grains of the rye sample used and fails to establish excised-root clones from the other cereals tested.

Addendum

Dr. A. Almestrand (Botanical Laboratory, University, Lund) in a private communication (8th September, 1954) informs us that he has also been successful in culturing excised rye roots. Using Burström's medium (Burström, 1941) containing either Difco or Cenovis yeast extract he has main-

tained his cultures during the last six months. Almestrand finds however that when sucrose is substituted for glucose in Burström's medium, growth of his root material is much reduced and soon stops so that subcultures cannot be made.

References

- Almestrand, A.: Studies on the growth of isolated roots of barley and oats. — *Physiol. Plant.* 2: 372. 1949.
- Growth factor requirements of isolated wheat roots (a preliminary report). — *Physiol. Plant.* 3: 293. 1950 a.
- Further studies on the growth of isolated roots of barley and oats. — *Physiol. Plant.* 3: 205. 1950 b.
- The effects of pyridoxine on the growth of isolated grass roots. — *Physiol. Plant.* 4: 224. 1951.
- Audus, L. J.: *Plant growth substances*. — Leonard Hill Ltd., London 1953.
- Avery, G. S. Jr. & Berger, J.: Tryptophane and phytohormone precursors *Science* 98: 513. 1943.
- Bennet-Clark, T. A. & Kefford, N. P.: Chromatography of the growth substances in plant extracts. — *Nature* 171: 645. 1953.
- Bentley, J. A. & Bickle, A. S.: Studies on plant growth hormones. II Further biological properties of 3-indolylacetonitrile. — *J. Exp. Bot.* 3: 406. 1952.
- Block, R. J., LeStrange, R. & Zweig, G.: *Paper Chromatography*. — Academic Press, New York, 1952.
- Bonner, J.: The production of growth substance by *Rhizopus suinus*. — *Biol. Zentr.* 52: 565. 1932.
- & Koepli, J. B.: The inhibition of root growth by auxins. — *Am. Journ. Bot.* 26: 557. 1939.
- & Bonner, H.: The B vitamins as plant hormones. — *Vitamins and Hormones*. 6: 225. 1948.
- Bowden, K. & Marion, L.: Biogenesis of alkaloids. V. Radioautographs of barley leaves fed with tryptophane - β - C^{14} . — *Can. J. Research* 29: 1043. 1951.
- Burström, H.: On formative effects of carbohydrates on root growth. — *Bot. Notiser.* 310. 1941.
- The influence of heteroauxin on cell growth and root development. — *Lantbruks-Högskolans Ann.* 10: 209. 1942.
- Duhamet, L.: Action de l'hétéro-auxine sur la croissance de racines isolées de *Lupinus albus*. — *Compt. rend. (Paris)* 208: 1838. 1939.
- Fiedler, H.: Entwicklungs- und reizphysiologische Untersuchungen an kulturen isolierter Wurzelspitzen. — *Zeit. Botan.* 30: 385. 1936.
- Galston, A. W.: Indoleacetic-nicotinic acid interactions in the etiolated pea plant. — *Plant. Physiol.* 24: 577. 1949.
- Glover, J.: Skatole as a growth-promoting substance. — *Nature* 137: 320. 1936.
- Gordon, S. A. & Wildman, S. G.: Conversion of tryptophane to a plant growth substance by conditions of mild alkalinity. — *J. biol. Chem.* 147: 389. 1943.
- Gustafson, F. G.: Tryptophane as an intermediate in the synthesis of nucleic acid by green plants. *Science* 110: 279. 1949.
- Harris, G. P.: Amino acids and the growth of isolated oat embryos. — *Nature* 172: 1003. 1953.

- Kandler, O.: Untersuchungen über die Wirkung von 2, 4-Dichlorphenoxyessigsäure, α -Naphthyllessigsäure, Natriumfluorid und Vitamin B₁ auf den Stoffwechsel in vitro kultivierter Maiswurzeln. — *Planta* 42: 304. 1953.
- & Vieregg, A.: Über den Einfluss von β -indolylessigsäure auf den Stoffwechsel in vitro kultivierter Maizwurzeln und Spargelsprosse. — *Planta* 41: 613. 1953.
- Kotte, W.: Wurzelmeristem in Gewebekultur. — *Ber. deut. botan. Ges.* 40: 269. 1922 a.
- Kulturversuche mit isolierten Wurzelspitzen. — *Beitr. allg. Botan.* 2: 413. 1922 b.
- Kulescha, Z.: Recherches sur la transformation du tryptophane sous l'action des tissus de Topinambour. — *Compt. rend.* 228: 1304. 1949.
- & Gautheret, R. J.: Recherches sur l'action du tryptophane sur la prolifération des cultures de tissus de quelques végétaux. — *Compt. rend. soc. biol.* 143: 460. 1949.
- Larsen, P.: The formation, occurrence and inactivation of growth substances. — *Ann. Rev. Plant. Physiol.* 2: 169. 1951.
- Lowe, J. S.: The enzymic absorption of sugars by excised roots. — *Proc. 7th Int. Bot. Congress Stockholm*. p. 753. 1950.
- McClary, J. E.: Synthesis of thiamin by excised roots of maize. — *Proc. Nat. Acad. Sci. U.S.A.* 26: 581. 1940.
- Mitchel, K. M.: Vitamins and metabolism in *Neurospora*. — *Vitamins and Hormones* 8: 127. 1950.
- Nagao, M.: Studies on the growth hormones of plants. III. The occurrence of growth substance in isolated roots grown under sterile conditions. — *Science Rep. Tohōku Imp. Univ. Fourth Series.* 12: 191. 1937.
- Studies on the growth hormones of plants. IV. Further experiments on the production of growth substance in root tips. — *Science Rep. Tohōku Imp. Univ. Fourth Series* 13: 221. 1938.
- Naylor, A. W. & Rappaport, B. N.: Studies on the growth factor requirements of pea roots. — *Physiol. Plant.* 3: 315. 1950.
- Pilet, P. E.: Répartition et variations des auxines dans les racines de *Lens culinaris* Medikus. *Experientia* 7: 262. 1951.
- Robbins, W. J.: Cultivation of excised root tips and stem tips under sterile conditions. — *Bot. Gaz.* 73: 376. 1922 a.
- Effect of autolized yeast and peptone on growth of excised corn root tips in the dark. — *Bot. Gaz.* 74: 59. 1922 b.
- Vitamin and amino acid requirements for the growth of higher plants. — *Plant Growth Substances*, Ed. F. Skoog. Univ. of Wisconsin Press, 1951.
- & Maneval, W. E.: Effect of light on growth of excised corn root tips under sterile conditions. — *Bot. Gaz.* 78: 424. 1924.
- Limited growth and abnormalities of excised corn root tips. — *Bot. Gaz.* 98: 209. 1936.
- & White, V. B.: Effect of extracts from the corn plant on growth of excised root tips. — *Bot. Gaz.* 98: 520. 1937.
- Skinner, J. C. & Street, H. E.: Studies on the growth of excised roots. II. Observations on the growth of excised groundsel roots. — *New Phytol.* 53: 44. 1954.
- Slankis, V.: Über den Einfluss von β -Indolylessigsäure und anderen Wuchsstoffen auf des Wachstum von Kiefernwurzeln. 1. — *Symbolae Botanicae Upsalienses* 11 (3). 1951.
- Street, H. E.: Factors controlling meristematic activity in excised roots. V. Effects of β -indolylacetic acid, β -indolylacetonitrile and α (1-naphthylmethylsulphide)-propionic acid on the growth and survival of roots of *Lycopersicum esculentum*, Mill. — *Physiol. Plant.* 7: 212. 1954 a.

- Growing roots without plants. — *Discovery* 15. 286. 1954 b.
- Factors controlling meristematic activity in excised roots. VI. Effects of various 'anti-auxins' on the growth and survival of excised roots of *Lycopersicum esculentum*, Mill. — *Physiol. Plant.* 8: 48. 1955.
- McGonagle, M. P. & Lowe, J. S.: Observations in the 'staling' of White's medium by excised tomato roots. — *Physiol. Plant.* 4: 592. 1951.
- & Roberts, E. H.: Factors controlling meristematic activity in excised roots. II. Experiments involving repeated subculture of the main axis meristem of roots of *Lycopersicum esculentum*, Mill. and *Lycopersicum pimpinellifolium*, Dunal. — *Physiol. Plant.* 6: 1. 1953.
- & McGregor, S. M.: The carbohydrate nutrition of tomato roots. III The effect of external sucrose concentration on the growth and anatomy of excised roots. — *Ann. Bot.* 16: 185. 1952.
- & Sussex, I. M.: Effects of 3-indolylacetic acid and 3-indolylacetonitrile on the growth of excised tomato roots. — *J. Exp. Bot.* 5: 204. 1954.
- Tatum, E. L. & Bonner, D.: Indole and serine in the biosynthesis and breakdown of tryptophane. *Proc. Natl. Acad. Sci. U.S.* 30: 30. 1944.
- Thimann, K. V.: On the plant growth hormone produced by *Rhizopus suinus*. — *J. Biol. Chem.* 109: 279. 1935.
- Van Overbeek, J.: Evidence for auxin production in isolated roots growing in vitro. — *Bot. Gaz.* 101: 450. 1939.
- & Bonner, J.: Auxin in isolated roots growing in vitro. — *Journ. Proc. Natl. Acad. Sci. U.S.* 24: 260. 1938.
- Wiltshire, G. H.: Oxidation of tryptophane in pea-seedling tissue and extracts. — *Biochem. J.* 55: 408. 1953.
- Würgler, W.: Über das Wachstum der Wurzeln von *Zea Mays* in Organkultur und seine Beeinflussung durch Wirkstoffe. — *Ber. Schweiz. Bot. Ges.* 52: 239. 1942.

Photoperiodic Cycles of Lengths Differing from 24 Hours in Relation to Endogenous Rhythms

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Introduction

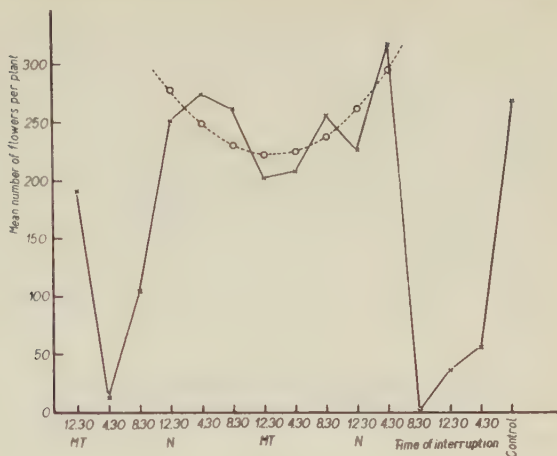
The question whether the daylength responses of plants are to be placed into the same category as other rhythmic phenomena has recently become prominent in discussions on the mechanism of photoperiodic reactions. The original suggestion and much work on this aspect are due to Bünning who has evolved a detailed hypothesis explaining the effects of daylength on the flowering of plants according to whether environmental changes are either in or out of phase with internally determined or endogenous rhythms. To some extent this theory consists of a restatement of the known observational facts in other terms. Bünning has however elaborated this hypothesis by suggesting that the phases in which light has a favourable effect are predominantly »synthetic», those in which it has not, predominantly »hydrolytic». The terms favourable and unfavourable are always used in relation to flower promotion or inhibition. The differential effect of light according to the phase in which it is given, is due to the inactivation of different substances: auxin in the photophile (favourable) phase and some substance stimulating flowering during the scotophile (unfavourable) phase. This last suggestion is however still regarded as tentative, (Bünning, 1952). The photophile and scotophile phases alternate according to Bünning's hypothesis following a regular sinusoidal curve with a period of 24 hours. On this basis it is possible to predict that a light break given during a long dark period (i.e. exceeding 12 hours) should promote or inhibit flowering according to

the phase during which it is given. This effect is well-known in short-day plants such as *Kalanchoë Blossfeldiana* grown in short day in a 24 hour cycle, where maximum inhibition results from a light break given near the middle of the dark period (Harder, 1948). In order to test Bünning's theory this observation was further extended by Claes & Lang (1947) with the long-day plant *Hyoscyamus niger* and by Carr (1952 a) with the short day plants *Perilla ocymoides* and *Xanthium saccharatum* to 48 hour cycles in which similar results were obtained, i.e. a light break given in the two scotophile phases of the long dark period was more effective than a break given during the photophile phase. However, Claes and Lang suggested an alternative explanation for these results; it seemed possible that the combination of the light break with the main light period preceding or following it actually constituted effectively a long light period, the intervening dark period being too short to have any effect. Since then Wareing (1954), has adduced further evidence that the effective light period is the sum of the main photoperiod plus intervening dark hours plus light break. In Biloxi soybean summation of these two light periods and intervening dark appears to take place if given in either order, while in *Xanthium* a light break following the main light period seems the more effective. In order to distinguish between these two alternative explanations Carr (1952 b), carried out a further experiment in which, after a twelve hour main light period, the dark was extended to 60 hours. This dark period embraced 3 scotophile phases and a light break given during the second of these was therefore preceded as well as followed by a long dark period and any unfavourable effect on flowering of a light break given at that time would argue against Claes and Lang's alternative suggestion. In this experiment Carr gave nine such 72 hour cycles and he obtained some flowering with light breaks in the first and second photophile phases while none of his plants budded when the light breaks fell into any of the three scotophile phases. Unfortunately this important result is marred by the fact that none of the controls i.e. those plants whose 60 hour dark period was not interrupted at all, appears to have flowered. Similarly only two treatments having breaks in the second photophile phase flowered compared with five in the first such phase and, as near as can be judged from the histogram, only 6 and 2 replicates out of 9 did so respectively.

In view of the importance of this experiment it was thought worth while to repeat it. This was done in the summer of 1953.

Methods and Results

The experiment was carried out during August and September in a well ventilated greenhouse whose temperature was not controlled, but which was kept low during



the 60 hour dark periods of each cycle by shading. In Carr's experiment the light-break treatments were at 3 hourly intervals; in the present experiment the interval was 4 hours, thus reducing the number of treatments from twenty to fifteen. The light break, of 30 minutes' duration, was given by means of incandescent light from two 12V 24W lamps suspended ab. 10 cm. above the plants which were arranged in a circle surrounding the lamps to exclude any mutual shading and to equalize light intensity. The lights were operated by a multiple time switch made from a thermograph clock and drum. Before the start of the experiment all axillaries were removed from the plants which were then sorted into twelve blocks according to initial size in order to minimize any subsequent variation in flower numbers etc. arising from such differences. Before and after the ten 72 hour cycles were given the plants were kept in continuous light (natural daylight prolonged by incandescent lamps). Apart from *Kalanchoë Blossfeldiana* as used by Carr some *Xanthium pennsylvanicum* plants were also included in this experiment, but in this case only four replicates were used and only three 72 hour cycles given.

Kalanchoë: Seven weeks after the end of the experimental treatments flower counts were made on the *Kalanchoe* plants and measurements of the inflorescences taken. The following data are presented in Figures 1—3: total number of flowers per plant, the mean reciprocal of the time to budding, and the plant heights.

As the main interest was centred on any effect during the second scotophile phase several statistical analyses were carried out on the eight treatments with light breaks ranging from the first to the second photophile phase in the 60 hour dark period, i.e. the treatments whose dark period was interrupted between noon of the second day and 4.30 p.m. of the third day. As will be seen from Figures 1—3, the effects of the light breaks in the first and third nights are so large as not to require statistical confirmation. Statistically significant differences between the other eight treatments were established on total flower numbers at the 1 per cent level when these treatments were grouped

Mere consideration of the number of plants flowering in the present experiment would not have shown any effect at all due to a light break in the second scotophile phase, but there is a noticeable inhibition in the first and third of these. The number of wholly vegetative plants with a light break at 4.30 a.m. in the first scotophile phase was five out of twelve, while eleven out of twelve remained vegetative with a light break at 8.30 p.m. and two out of twelve did so with a light break at 12.30 midnight in the third scotophile phase. From this it would seem rash to conclude that Bünning's explanation is the only one applicable and that Claes and Lang's alternative must be rejected as inadequate. A more likely interpretation seems to be that both may in fact apply. Bünning's effect due to internal rhythms would seem to be relatively slight compared with the effective long day produced by the combination of main light period and light break. Although temperatures were not maintained constant in the present experiment but underwent regular diurnal variations in the greenhouse, it would seem unlikely that the whole of the effect described could be caused by such changes.

Experiments with cycles of different total lengths carried out in Lappland

From experimental results reported by Schmitz (1951), who used a wide range of cycle lengths with different dark and lights periods, and whose data are discussed below, it would also seem that endogenous rhythms alone cannot suffice to explain the reaction of short-day plants. A similar experiment to Schmitz's was carried out by the author in Lappland in the summer of 1952, taking advantage of the continuous daylight prevailing during the Arctic summer. This made it possible to combine light and dark periods factorially into cycles of greater and smaller total lengths than the normal twenty four hours without having to use artificial sources of illumination. In order to spread the diurnal variations in light intensity (which are of course quite marked) over all the cycles, multiples of five hours were chosen so that none of the cycles coincided with the normal day. Hence these fluctuations of light intensity were not confounded with treatments. The following periods were chosen: light 5, 10 and 20 hours, dark 10, 20, 30 hours giving nine different cycles. These were given for twelve induction periods only, all plants receiving continuous daylight before and after such treatment. The number of induction periods was limited by the time available in the Arctic for experimentation, and as twelve such periods of the longest cycle represented twenty five days it was not possible to exceed this. Six replicates each of three short day species were grown in this experiment: *Kalanchoë Blossfeldiana*, *Xanthium pennsylvanicum* and a short-day variety of *Impatiens balsamina*. The results are shown in Table 2.

Table 2. *Flowering etc. of three short day species in cycles combining 5, 10 and 20 hours light factorially with 10, 20 and 30 hours dark. Twelve such induction cycles were given preceded and followed by continuous daylight. Six replicates were used in each treatment.*

Hours light Hours dark	L 5			L 10			L 20		
	D 10	D 20	D 30	D 10	D 20	D 30	D 10	D 20	D 30
<i>Kalanchoë:</i>									
No. of plants budded	0	6	5	0	6	6	0	2	0
Mean No. of flowers in terminal inflorescence	0	143.3	10.1	0	80.1	79.7	0	2	0
Mean leaf pair increment to inflorescence ¹ ..	5.0	2.5	4.0	5.7	2.9	3.0	5.1	5.5	5.1
Mean height increment at budding cms ¹ ..	8.0	3.7	4.4	6.6	4.5	5.1	7.3	7.1	8.4
Days to budding	—	19.0	32.5	—	22.8	24.5	—	33.0	—
Mean length of main infl. axis cms	—	5.7	1.3	—	3.9	3.9	—	1.8	—
<i>Xanthium:</i>									
No. of plants budded	2	6	6	6	6	6	6	6	6
Days to budding	34.0	14.3	17.1	14.0	15.5	15.1	16.0	14.3	14.0
Leaf No. increment ¹	8.8	5.0	5.1	5.5	5.3	5.1	5.7	5.3	5.7
Height increment to budding cms ¹	14.2	2.4	2.3	5.0	4.5	2.7	5.8	4.6	5.1
<i>Impatiens:</i>									
No. of plants budded	0	6	0	0	6	5	0	6	6
Days to budding	—	23.0	—	—	31.0	36.8	—	31.5	31.3
No. of axil with first bud	—	7.7	—	—	8.3	10.0	—	10.0	10.7

¹ Values for vegetative plants are those attained at the end of the experiment.

In *Xanthium* flowering occurred in eight out of the nine cycles given, the only exception being the shortest cycle combining a dark period of 10 hours with 5 hours light, in which only two plants budded very late, the other four replicates remaining quite vegetative. This failure cannot be said to be due entirely to the shortness of the dark period for the same dark period combined with 10 or 20 hours light caused flower initiation. On the other hand 5 hours light are not limiting flowering if combined with longer dark periods such as 20 or 30 hours dark. It would seem that this result is similar to those noted by Wareing (unpubl.) who found for Biloxi soy bean that the critical dark period is increased as the photoperiod is shortened. Again both these results may be related to a recent observation (Schwabe, 1954), that the critical dark period increases as the light intensity preceding the dark period is decreased and a very short photoperiod may have the same effect as one of insufficient intensity.

In *Balsam* 10 hours dark was insufficient to cause flowering regardless of the length of the light period; 5 hours light combined with 30 hours dark caused a high degree of etiolation and probably for this reason again no flower initiation occurred. All other combinations of light and dark led to flower initiation.

In *Kalanchoë* again 10 hours dark was too short for any flower initiation. In this species as in *Xanthium* flowering did take place in 5 hours light plus 30 hours dark. In *Kalanchoë* however, 20 hours light proved to be too long a light period for successful flower initiation and only in combination with 20 hours dark were two plants induced to produce any flowers at all. When 20 hours light were combined with 10 or 30 hours dark all six replicates remained completely vegetative. In general these results with *Kalanchoë* agree very well with the data gathered by Schmitz (1951) the only exception being a cycle of 10 hours light plus 10 hours dark — here vegetative — for which he quotes a result in the literature indicating flowering.

The successful flowering then of *Xanthium* in cycles with total lengths of: 20, 25, 30, 35, 40 and 50 hours; of *Impatiens* in cycles of: 25, 30, 40 and 50 hours; and *Kalanchoë* in cycles of: 25, 30, 35 and 40 hours would again make it appear unlikely that the prime cause of photoperiodic responses must be sought in the alternation of phases of an internal rhythm which must largely coincide with the appropriate changes of light and dark in the external environment. Even if some adaptation to cycles other than twenty-four hour cycles is postulated, some limit must be set to this or the rhythm can no longer be regarded as endogenous at all. It seems hard to believe that adaptation occurred in all the above cycles which led to successful flowering.

In the case of *Kalanchoë* the results shown in Figure 25 of Schmitz's paper suggest a relatively simple relation between the light and dark periods suitable for flowering, i.e. there is, apart from the well-known minimum or »critical» dark period, also a maximum light period which will allow flowering to occur and if this is exceeded the vegetative condition will be maintained regardless of the associated dark period. Possibly this may represent some over-simplification since Wareing's (1953) results would indicate that the critical dark and light periods are not absolutely independent and that interactions occur. Failure to flower in spite of an adequate dark period but when the effective light period given was too long has also been demonstrated by Wareing (1953).

Discussion and Conclusions

The repetition of Carr's important experiment (1952 b) in which a 72 hour cycle was used has yielded results which tend to confirm that rhythmic changes in the plant may affect its sensitivity to light breaks given in the course of a long dark period, as postulated by Bünning. For it is improbable that the above findings can be ascribed entirely to diurnal temperature changes during the experiment, both since they support Carr's results, which were obtained with constant temperatures, and in view of Harder's demon-

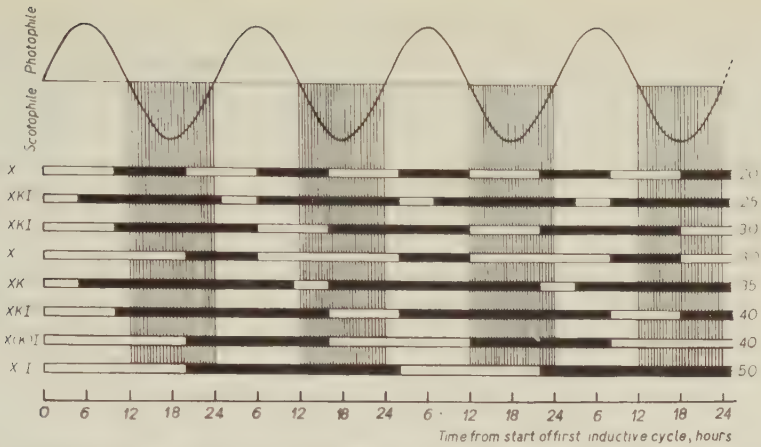


Figure 4. Photoperiodic cycles which led to successful flowering in X=*Xanthium*, K=*Kalanchoë*, and I=*Impatiens*. The length of one light plus one dark period (in hours) is shown at the end of each line. The cycles are shown for four consecutive periods of 24 hours together with the photophile and scotophile phases of a diurnal endogenous rhythm (for short-day plants) starting at the beginning of the first inductive cycle.

stration of the absence of temperature effects on this response (Harder et al., 1944). However, in the present experiment the magnitude of the effect was much smaller than in Carr's data. A possible explanation for this discrepancy may perhaps be found in the different states of the plants in the two experiments particularly in view of the complete failure of Carr's controls to flower with an uninterrupted dark period of 60 hours. Again the small degree of flowering in those of his treatments in which the light break coincided with the second photophile phase also suggests that his plants were less vigorous than those used in the present experiment. Hence the adverse effects on flowering which he records would seem to be exaggerated making it difficult to assess their relative importance in more normal circumstances.

The uniform flowering of *Xanthium pennsylvanicum* in the present experiment throughout all light break treatments and controls, however, does not suggest that any rhythmical processes are concerned in this species. Similarly the much smaller effect of light breaks on *Kalanchoë* in the second scotophile phase compared with the first and third also seems to indicate that the effects of internal rhythms are not of a magnitude sufficient to account for all photoperiodic responses.

Further, those experiments involving nine different factorial combinations of daylight and dark also do not suggest that an internal 24-hour-rhythm can be the main controlling factor in flowering. In all three species investigated flowering occurred in cycles differing widely from 24 hours, and the fact

that the periods employed were multiples of 5 hours ensured that coincidences with a 24-hour period were quite infrequent. This is illustrated in Figure 4, where only those cycles which led to flowering in one or all of the three species are shown, together with a 24-hour rhythm starting at the beginning of the experiment. Except where plants became rather etiolated and are therefore likely to have remained vegetative for this reason, failure to flower could be attributed either to the dark period being too short, or to excessive length of the light period (20 hours in *Kalanchoë*). If »adaptation» accounts for flowering in all these instances, the internal rhythm must be equally adjustable to a wide variety of cycle lengths.

In view of these facts it is necessary to reconsider Bünning's theory in some detail. The main difficulty in this hypothesis lies in the lack of definition of the term »endogenous» as it is applied to the rhythms held responsible. In a recent paper Bünning (1954) stresses the fact that rhythmic changes in leaf movements have been under discussion for more than 50 years, and were not invented to explain photoperiodism. His own chief contribution to the subject has been to attribute photoperiodic effects to the same causes; i.e. photoperiodic responses are to be regarded as manifestations of the phenomenon of an internal rhythm interacting with an environmental rhythm. Thus although not invented for the purpose it is claimed that these internal rhythms must be taken into account in order to explain photoperiodic phenomena. If this is so it would seem that a causal relation should be established — a mere correlation between the two phenomena of leaf movements and flowering responses would not in itself serve for this purpose unless the physiological causes of leaf movement were known — which is not the case. In the paper referred to, Bünning gives a further exposition of his hypotheses relating photoperiodism to these endogenous rhythms, and three of his statements may be quoted here: — (a) »Behauptet und bewiesen wird nur, dass es sich bei alledem um nicht anderes handelt, als um die endogene Tagesrhythmik, bei der eben bestimmte Phasenlängen erblich festgelegt sind, und die durch Licht und Dunkelheit regulierbar und modifizierbar ist.» (b) »Muss denn eigentlich immer wieder auf das einfache Beispiel des Pendels hingewiesen werden, dessen Schwingungsdauer auch endogen, nämlich durch die Pendellänge, festgelegt ist, und bei dem ebenfalls die Lage der Phasen willkürlich durch neue Anstösse modifiziert werden kann?» (c) »Aus diesen Ergebnissen folgt also, dass bei der Soja, die im normalen Licht-Dunkel-Wechsel des Gewächshauses aufgezogen ist, ein einzelner Lichtreiz nur ausreicht, um die endogene Tagesrhythmik für etwa 30 Stunden einzuregulieren: Der Lichtreiz induziert eine photophils Phase (Gesamtdauer etwa 10 Stunden), eine anschliessende skotophile (Gesamtdauer etwa 14 Stunden), und eine

nochmalige photophile Phase, die aber schon abgekürzt sein kann. Dann beginnt die Dunkelstarre.»

The endogenous component of such rhythms is thus to be regarded as analogous to a pendulum whose length is »*endogenously fixed*», (hereditarily determined lengths of phases), but which may be set in motion by external agencies, the important characteristic being the length of the phases and not merely an alternation of maxima and minima which may be continued even in the absence of external stimuli.

However, Bünsow (1953 a) has pointed out that the term »endogenous diurnal rhythm» itself has been used in several different senses and he makes the following distinctions: — »1. die endogen-tagesrhythmischen Bewegungen, 2. die Rhythmik dieser Bewegungen und 3. die inneren Ursachen dieser Bewegungen, hier das endodiurnale System genannt.» However, it is not known whether Bünning has accepted these distinctions.

As regards leaf movements Bünsow states that the term *endogenous* refers to the resultant of both external and internal causes. At the same time he opposes a strict distinction between external and internal effects, which he prefers to treat as »polar» concepts — though this does little to clarify our ideas concerning the mechanism of endogenous rhythms.

In order to justify a theory that photoperiodic responses are explained by an endogenous rhythm which is either subject to environmental modification (Bünning) or the resultant of external and internal factors (Bünsow), two requirements must be fulfilled by these rhythms: —

- 1) they must be shown actually to control flowering behaviour, and
- 2) they must allow for adaptation to cycles of light and dark whose total duration differs from the 24 hours normally accepted as the period of the natural rhythm.

We may confine our attention to short day plants, since in long-day plants the position is still that continuous light without any alternation of phases is usually optimal for flowering. If we accept Bünsow's definition it may be assumed the »endodiurnal system» retains its fixed phase length once it is set in motion and is not affected by the environment, an idea which also would agree with Bünning's simile of a pendulum of fixed length. The actual phase lengths of the observed phenomena would then be the resultant of the fixed internal rhythm and the rhythm superimposed by the environment. In cases of »adaptation» to cycles widely differing from 24 hours the environmental effect must be regarded as completely dominant and obliterating the effects of the internal autonomous endodiurnal system. It would seem very necessary therefore, to devise subsidiary hypotheses to account for the fact that »adaptation» is not possible to all kinds of cycle lengths and to explain

in what sense »failure to adapt» should be regarded as re-assertion of the internal rhythm.

On the other hand it might well be that under constant external conditions a natural autonomous rhythm might become apparent. In contrast to leaf and flower movements however, we have no means of detecting the hypothetical alternation of sensitivity to light and dark in the case of flowering responses. The only criteria are the eventual success or failure of floral initiation, its intensity and the time taken before it is achieved, and in order to detect any effect at all we have to impose some light/dark regime which may or may not interfere with the rhythm under investigation. Hence control of flowering by the internal rhythm could be effective only under exceptional conditions such as cycles with very long light or dark periods in which the imposed effects of external stimulation have faded out.

If on the other hand we do not follow this interpretation of Bünsow's remarks in regarding the internal unobservable rhythm as fixed and distinct from the rhythm manifested, then we may treat the rhythm of leaf movement and the internal rhythm as identical. To return to Bünning's simile, once the pendulum of fixed length has been set in motion the endogenous rhythm is also fixed and the flowering response of short-day plants will depend on whether, after the initial light period, a subsequent light period or short light break falls into a photo- or scotophile phase as these succeed one another. But this would preclude any adaptation to cycles of total lengths other than 24 hours or multiples thereof, and hence some of those shown in Fig. 4, in which normal flowering occurred, clearly could not be accounted for on this hypothesis: nor for that matter could many of the leaf movements recorded by Kleinhoonte (1929) and quoted by Bünning (1953). A way may be found out of this dilemma in the case of long period cycles if the persistence of the endogenous rhythm is found to be limited in darkness, so that after a short period the oscillation fades out, e.g. in the paper cited it is claimed that under these circumstances the endogenous rhythm in soy bean ends after 30 hours, and that once dark rigor of leaf movement has set in a new light stimulus is required to start it again. If the rhythm is thus damped completely between two periods of endogenous oscillation it is possible on the theory to accommodate effective cycles having excessively long dark periods (and possibly something similar might be postulated for cycles containing long light periods), but the problem of adaptation to cycles of total lengths less than 24 hours still remains unsolved. Also, if dark rigor is postulated after say 30 hours, how are experiments like Carr's 72 hour cycle, and its repetition reported above, to be accounted for? A stage of indifference before the next light stimulus — dark rigor — would then be reached after 30 hours, and beginning from the middle of the second scoto-

phile phase any light break would merely initiate another endogenous oscillation and could only be unfavourable if so near to the next main light period as to make that fall into a scotophile phase. Instead of »*proving*» Bünning's hypothesis this experiment would already be difficult to reconcile with it, though one might assume that in *Kalanchoe* the endogenous rhythm is not extinguished as rapidly as in soybean and may extend for more than 30 hours. Experiments with yet longer dark periods than 60 hours would then be needed to test the applicability of this subsidiary hypothesis of dark rigor, but the longer the interval before dark rigor sets in the more difficult would it be to use this concept to explain adaptation to cycles with more moderately long dark periods.

The only way to account for adaptation — especially of course with regard to short cycles — would then be to make the length of the pendulum itself variable, but thereby the fixed periodic character of the rhythm would be abandoned. To say that the »length» of the pendulum has not been altered, but that its motion has been affected by the environment, e.g. arresting or accelerating it during *each and every* swing, would merely be making unverifiable assumptions since our only knowledge of the assumed *fixed* length of the pendulum is derived from its period.

Bünning states that in relation to flowering it is necessary to study first what »endogenous» rhythm the plant may adopt in a particular set of circumstances, as in the soybean example given; the theory would not therefore help us to predict flowering behaviour without prior experimentation. At best it would confirm a strong correlation between leaf movements (and presumably also flower movements as studied by Bünsow) and flowering, but this need have no greater significance than other similar correlations, such as those with leaf succulence in *Kalanchoë*, tuberization in the artichoke etc., and knowing one the other might be predicted. Any precise correlation between the processes underlying flower induction which so far are not open to direct observation and leaf movements which are directly observable would be of great value in the analysis of flowering phenomena but to be of general application an invariable correlation of this kind must be established. However, even this correlation does not seem to be too well established. The fact that conditions which would not lead to the elimination of photoperiodic responses can in fact easily upset the regularity of rhythmic leaf movements can be seen from Bünning's own paper (1948), in which he remarks that in two of three greenhouses used for experiments on Maryland Mammoth Tobacco, because of shading and of insufficiently great temperature differences prevailing between day and night, the movements became irregular, (», . . die Bewegungen wurden uneinheitlich .) His further discussion of results was then confined to those treatments giving the simple

correlative in the third house. Bünsow (1953b) also reports that flower movements in *Kalanchoë* can be made to follow an 8 hours light and 8 hours dark rhythm, and even one consisting of 6 hours each of light and dark, i.e. the endogenous rhythm will adapt itself to these regimes. Nonetheless there is no flowering of *Kalanchoë* in either of these two cycles (cf. also Schmitz, 1951). Hence adaptation is possible, here at least, for but one of these two correlated characteristics!

The intervention of an endogenous rhythm which is itself regulated by the environment and in its turn controls flowering therefore amounts to an unnecessary postulate and direct action of light and dark stimuli on the system leading to flowering provides a simpler hypothesis.

The recent observation of Gregory et al. (1954) that carbon dioxide fixation in the dark appears to be closely linked with photoperiodic responses allowed another factor showing maxima and minima to be examined for the persistence of its rhythm and it was shown that in either continuous light or dark a steady state is reached without any oscillation.

In addition in *Kalanchoë* at least we have to consider the inhibitory effect of long days intercalated between favourable short days (Schwabe, in the press). These experiments demonstrate that the effects of individual »photoperiodic cycles» comprising one light and one dark period do not merely depend on the conditions prevailing within that cycle but can be modified by the conditions in the preceding cycle. Such interactions would present even greater difficulties in any appeal to endogenous rhythms as an »explanation» of photoperiodic phenomena.

It would seem then that with rhythmic changes of the environment we can induce rhythmic changes in leaf movements etc.; when continuous conditions are given, rigor (light or dark) will ensue, but before this sets in (i.e. before the »steady state» is reached) a number of oscillations — frequently with a period of 24 hours — will take place, much in the same way as for example geotropic and phototropic growth responses lead to overcompensation and oscillation. Oscillations of this kind, even though their period is determined by internal rhythmic changes of metabolism in the absence of external stimuli, would not seem to »put our ideas of photoperiodism into a wider framework» for there is no substantial evidence that the internal rhythm represents the main controlling factor in photoperiodic responses; on the other hand under special circumstances the rhythm might tip the balance in one or other direction in the same way as other internal factors do.

In view of the present divergence of opinions on the endogenous character of alternations in the metabolism of plants and their relation to flowering behaviour, it would be of real value if the theory could be expounded in

more concrete terms, and especially if Professor Bünning would define the sort of experimental result which he would regard as incompatible with it.

Summary

The effects of light breaks on the flowering of *Kalanchoë Blossfeldiana* when given at different times during the 60 hour dark period of a 72-hour cycle are described. This repetition of Carr's experiment to test Bünning's hypothesis of photoperiodism indicated that there was a significant reduction in flower numbers when the light breaks coincided with Bünning's three scotophile phases, but the reduction during the second of these — though statistically significant — was only slight. Under the same conditions *Xanthium pennsylvanicum* flowered in all treatments.

In another experiment carried out in Arctic Lappland three short-day species (*Xanthium*, *Kalanchoë*, and a short-day variety of *Impatiens balsamina*) were given photoperiodic cycles comprising nine factorial combinations of daylight and dark. The total cycle lengths of these combinations ranged from 15 to 50 hours and consisted of multiples of 5 hours to avoid coincidences with a 24-hour cycle. In this experiment flowering occurred in cycles whose lengths differed widely from 24 hours and (except where plants became rather etiolated and are therefore likely to have remained vegetative for this reason) failure to flower could be attributed either to the dark period being too short or (in *Kalanchoë*) to excessive length of the light period.

The results are discussed critically in relation to Bünning's hypothesis of endogenous diurnal rhythms which, it is concluded, cannot account for all photoperiodic responses.

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References

- Bünning, E.: Weitere Versuche über die Beziehung zwischen endogener Tagesrhythmik und Photoperiodismus. — Z. f. Naturf. 3 b: 457. 1948.
 — Morphogenesis in Plants. — Survey of Biological Progress 2: 105. 1952.
 — Entwicklungs- und Bewegungsphysiologie der Pflanze. Springer Verlag, Berlin, 1953.
 — Der Verlauf der endogenen Tagesrhythmik bei photoperiodischen Störlicht-Versuchen mit Soja. — Physiol. Plant. 7: 538. 1954.

- Bünsow, R.: Über den Einfluss der Lichtmenge auf die endogene Tagesrhythmik bei *Kalanchoë Blossfeldiana*. — *Biol. Zentralbl.* 72: 465. 1953 a.
- Endogene Tagesrhythmik und Photoperiodismus bei *Kalanchoë Blossfeldiana*. — *Planta* 42: 220. 1953 b.
- Carr, D. J.: The photoperiodic behaviour of short-day plants. — *Physiol. Plant.* 5: 70. 1952 a.
- A critical experiment on Bünning's theory of photoperiodism. — *Z. f. Naturf.* 7 b: 570. 1952 b.
- Claes, H. & Lang, A.: Die Blütenbildung von *Hyoscyamus niger* in 48-stündigen Licht-Dunkel-Zyklen und in Zyklen mit aufgeteilten Lichtphasen. *Z. f. Naturf.* 2 b: 56. 1947.
- Gregory, F. G., Spear, I. & Thimann, K. V.: The interrelation between CO_2 metabolism and photoperiodism in *Kalanchoë*. — *Plant Physiol.* 29: 220. 1954.
- Harder, R.: Vegetative and reproductive development of *Kalanchoë Blossfeldiana* as influenced by photoperiodism. — *Symp. Soc. Exp. Biol.* II. Growth, 117. 1948.
- Wallrabe, E. & Quantz, L.: Über die Rolle der Temperatur bei der Zerstörung des Blühimpulses durch Zwischenbeleuchtung. — *Planta* 34: 41. 1944.
- Kleinhoonte, A.: Über die durch das Licht regulierten autonomen Bewegungen der *Canavalia*-Blätter. — *Arch. néerld. Sci. exp. et natur.* (Ser. III b) 5: 1. 1929.
- Schmitz, J.: Über Beziehungen zwischen Blütenbildung in verschiedenen Licht-Dunkelkombinationen und Atmungsrythmik bei wechselnden photoperiodischen Bedingungen. — *Planta* 39: 271. 1951.
- Schwabe, W. W.: The effect of light intensity on the flowering of *Kalanchoë Blossfeldiana* in relation to the critical daylength. — *Physiol. Plant.* 7: 745. 1954.
- Evidence for a flowering inhibitor produced in long days in *Kalanchoë Blossfeldiana*. — *Ann. Bot.* (in press).
- Wareing, P. F.: A new photoperiodic phenomenon in short-day plants. — *Nature* 171: 614. 1953.
- Experiments on the »light-break» effect in short-day plants. — *Physiol. Plant.* 7: 157. 1954.

The Effect of Auxins on the Growth and Yield of Wheat

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Cholodny (1936) was perhaps the first to claim that seeds treated with indolylacetic acid produced plants that yielded substantially more than plants from untreated seeds. Since then numerous attempts have been made to stimulate the growth and yield of plants by treating either seeds or growing plants with auxins. The literature on the 'hormonization' of seeds has been exhaustively reviewed by Kruyt (1954) and that on treatment of growing plants by Pearse (1948). It appears that there is little unanimity of opinion about the beneficial effect of application of auxins on the growth of the whole plant. The interest of the writers in the study of this problem was, however, stimulated by the observation of Leopold and Thimann (1948) that low concentrations of auxins increased the number of flower primordia in Wintex barley when subjected to an appropriate photoperiod. The writers undertook this study with wheat in order to see if the increase in spikelet number, resulting from auxin application, also led to increase in grain yield. This paper describes the results of experiments carried out during five seasons.

Experimental Procedure

The plants were raised in earthen garden pots, each having capacity to hold about 22 lb of air-dry soil. Seeds were sown on the 14th November in every season (optimum sowing time). Six uniform seedlings were ultimately retained per pot, there being five pots per each treatment. The soil was taken from a field lying fallow in the previous season and was well mixed with about 12 oz of well-rotted farm yard manure (per pot). A fortnight after sowing, two gm each of ammonium sul-

phate, superphosphate and potassium sulphate were added per pot. Another dose of 2 gm of ammonium sulphate was applied at the boot-leaf stage as it was found to improve growth. Farmyard manure and ammonium sulphate were omitted when relative nitrogen deficiency was included as a treatment.

Auxin was infiltrated into the plant through cut leaf tip in the manner described by Leopold and Thimann (1948). In the first three seasons only this technique was adopted; in the fourth season spray application of auxin solution was also included and in the fifth season spraying alone was carried out. Water was applied as spray or through leaves to the controls. The cut leaf tips of the youngest mature leaves of two neighbouring plants were dipped into one c.c. solution. This quantity was absorbed by the two plants within about six weeks — a much longer time than that reported by Leopold and Thimann (1948) and Hussey and Gregory (1954). The treatments commenced about three weeks after sowing, the growing point at this stage having elongated prior to differentiation of double ridges. 15 c.c. of solution was applied as spray per pot, with a repetition a week later. In the fifth season a small scale field trial was also carried out.

The pots were kept in the open throughout the season, exposed to the natural variations of day-length and temperature. The data were statistically examined by the analysis of variance.

Experimental Results

The results obtained in the first three seasons are summarised in table 1. In these experiments beta-indolylacetic acid (IAA) was used. Only in one season was the yield significantly increased, the effect being due to increase in ear number. Other characters were also favourably or unfavourably influenced in some seasons. In none of these seasons was the mean spikelet number per plant increased; the spikelets of the main axis were, however, not counted separately.

1952—53 Experiment

In the 1952—53 season three other substances namely, alphanaphthalene acetic acid (NAA), beta-indolylbutyric acid (IBA) and triiodobenzoic acid (TIBA), besides IAA, were used. Only two varieties, N.P. 710 and Punjab C 228 were included in the experiment. Some of the treatments significantly increased grain yield, tiller production, ear number and leaf as well as spikelet number on the main shoot (tables 2 and 3). None of the treatments had any effect on grain number per ear, 1000-grain weight, mean spikelet number per plant and time of anthesis. The significant effects on yield and ear number are summarised in table 4. In N.P. 710, in 5 cases out of 11, the increase in grain yield was due to increase in ear number. Inspection of the data on grain number per ear and 1000-grain weight revealed that these two characters were favourably influenced under the other six treatments, although not sig-

Table 1. *Effect of IAA during the first three seasons.*

Variety	Conc. p.p.m. IAA	Tiller Number	Ear Number	Spikelet Number	Grain Number	1000 grain weight	Grain yield
1949—50							
Punjab							
C 228	10	0	+	0	0	0	+
„	20	0	+	0	0	0	+
S 40	10	+	+	0	0	0	+
„	20	0	0	0	0	0	0
(No effect of 1 and 100 p.p.m. IAA.)							
1950—51							
Punjab							
C 228	10	0	0	0	—	0	?
Agra Local	10	0	+	0	0	0	?
(No effect of 200 p.p.m. IAA: no effect on varieties N.P. 52 & N.P. 710.)							
1951—52							
N.P. 165	20	—	0	0	0	0	0
„	200	—	0	0	+	—	0
N.P. 52	20	0	+	0	0	0	0
„	200	0	0	0	0	0	0

(No effect on varieties Agra Local and Punjab C 228)

0=no effect; —=depression; +=increase.

?=Plants damaged by hail before harvest; yield not recorded.

nificantly, and thus their combined influence increased grain yield. In Punjab C 228, the increase in yield due to treatments was completely accounted for by increase in ear number.

1953—54 Experiment

In 1953—54, interaction of nitrogen supply and hormone spray on varieties, N.P. 710 and Punjab C 228, was studied. Only two substances, NAA and IAA, were used at two concentrations, 75 and 100 p.p.m. each. Differential nitrogen supply was obtained by omitting farmyard manure and ammonium sulphate; the deficiency of nitrogen was quite apparent from the usual symptoms and the low level of grain yield. The data for N.P. 710 are given in table 5 and for C 228 in table 6. It will be seen that only IAA at both concentrations significantly increased grain yield of N.P. 710 and that this increase was very largely due to increase in grain number per ear. It may be noted that the mean spikelet number per plant was not increased by any treatment. The interaction between nitrogen and auxin was significant in the case of grain number per ear, the latter being differentially influenced by NAA and IAA at the two levels of nitrogen. It is not possible to account for these differential effects. Both

Table 2. *Effect of Auxins on N.P. 710 — 1952—53.*

p.p.m.	Auxin	Grain yield in gm/pot	Spikelet number per ear	Leaf number	Shoot number per pot	Ear number per pot
Through leaf		(Main axis only)				
10	IAA	40.7	18.9	10.0	48.2	31.0
15	»	43.0	18.8	10.3 ¹	47.8	31.6
20	»	39.4	19.2	10.1 ¹	47.2	29.0
30	»	42.4	19.2	10.4 ¹	47.0	29.8
10	NAA	43.7	19.2	9.9	45.8	31.0
15	»	47.6 ¹	19.3	9.6	47.6	33.4
20	»	45.3 ¹	19.5	10.3 ¹	54.2	31.6
30	»	57.5 ¹	20.3 ¹	9.8	59.0	37.0 ¹
Spray						
75	IAA	43.0	19.5	10.5 ¹	57.4	32.6
100	»	47.0 ¹	19.8 ¹	10.7 ¹	63.2 ¹	35.4
200	»	39.6	19.8 ¹	10.3 ¹	62.4 ¹	31.4
75	NAA	48.9 ¹	19.8 ¹	10.8 ¹	59.0	36.6 ¹
100	»	45.3 ¹	19.9 ¹	10.5 ¹	58.2	35.2
200	»	41.0	19.5	10.6 ¹	52.4	33.4
75	IBA	50.3 ¹	19.7 ¹	10.8 ¹	59.4 ¹	35.6 ¹
100	»	49.3 ¹	19.8 ¹	10.8 ¹	58.6	37.2 ¹
200	»	38.5	19.8 ¹	10.5 ¹	51.4	29.2
75	TIBA	50.9 ¹	20.2 ¹	10.6 ¹	57.0	37.6 ¹
100	»	49.0 ¹	19.8 ¹	10.4 ¹	55.8	34.6
200	»	47.1 ¹	20.0 ¹	10.4 ¹	55.4	34.2
Control		37.9	19.1	9.5	52.2	31.2
S.D. (5 % P)		7.3	0.5	0.5	6.9	4.2

¹ Values significantly greater than control.Table 3. *Effect of Auxins on Punjab C 228 — 1952—53.*

p.p.m.	Auxin	Grain yield in gm/pot	Spikelet number per ear	Leaf number	Shoot number per pot	Ear number per pot
Through leaf		(Main axis only)				
10	IAA	43.9	15.5	10.7 ¹	60.6	35.4
15	»	37.1	15.4	10.9 ¹	56.2	30.8
20	»	58.2 ¹	16.1	10.6 ¹	68.2 ¹	40.2 ¹
30	»	54.9 ¹	15.8	10.7 ¹	74.4 ¹	42.8 ¹
10	NAA	60.5 ¹	16.4 ¹	11.2 ¹	70.8 ¹	42.6 ¹
15	»	52.6 ¹	16.0	10.7 ¹	64.8	40.2 ¹
20	»	63.4 ¹	16.9 ¹	10.7 ¹	73.0 ¹	42.0 ¹
30	»	48.9 ¹	16.0	10.2 ¹	61.8	35.6
Spray						
75	IAA	42.9	15.7	10.9 ¹	63.4	35.4
100	»	39.7	15.7	10.3 ¹	59.2	32.4
200	»	32.6	15.3	10.5 ¹	57.2	29.4
75	NAA	57.5 ¹	16.6 ¹	10.4 ¹	64.0	39.6 ¹
100	»	54.0 ¹	16.3	10.2 ¹	68.6 ¹	39.4 ¹
200	»	40.6	16.0	10.4 ¹	58.2	34.4
75	IBA	40.5	15.9	10.5 ¹	58.8	33.0
100	»	40.1	15.9	10.2 ¹	52.2	31.8
200	»	36.7	15.8	10.3 ¹	51.2	29.2
75	TIBA	40.2	15.9	9.7	57.6	34.4
100	»	41.5	15.7	10.3 ¹	52.4	32.0
200	»	36.0	15.9	10.2 ¹	53.8	31.6
Control		35.1	15.6	9.6	56.8	30.0
S.D. (5 % P)		10.4	0.7	0.3	8.7	5.7

¹ Values significantly greater than control.

Table 4. *Summary of significant effects on yield and ear number (1952-53).*

N.P. 710											
Through Leaf			Spray								
	NAA	NAA	NAA	IAA	NAA	NAA	IBA	IBA	TIBA	TIBA	TIBA
p.p.m.	15	20	30	100	75	100	75	100	75	100	200
Yield	+	+	+	+	+	+	+	+	+	+	+
Ears			+		+		+	+	+		

Punjab C 228									
Through Leaf						Spray			
	NAA	NAA	NAA	NAA	IAA	IAA	NAA	NAA	
p.p.m.	10	15	20	30	20	30	75	100	
Yield	+	+	+	+	+	+	+	+	
Ears	+	+	+	+	+	+	+	+	

Table 5. *Effect of auxins and nitrogen on N.P. 710 — 1953-54.*

Mean grain yield in gm per pot	Control	IAA 75	IAA 100	NAA 75	NAA 100 p.p.m.	
N ₁ ¹	26.3	34.8	31.9	29.3	29.6	30.4
N ₂ ¹	37.4	54.8	67.5	37.8	45.7	48.6
	31.9	44.8	49.7	33.6	37.7	

(Mean effects of nitrogen and auxins significant. S.D. (5 % P) for nitrogen, 7.9; for auxins, 12.5.)

Mean grain number per ear	Control	IAA 75	IAA 100	NAA 75	NAA 100 p.p.m.	
N ₁	28.2	34.6	32.0	32.3	31.6	31.8
N ₂	33.0	39.2	41.1	34.5	36.7	37.1
	30.6	36.9	36.6	33.7	34.2	

(Mean effects of nitrogen, auxins and interaction significant. S.D. (5 % P) for nitrogen, 1.3; for auxins, 2.2; for interaction, 2.9.)

Mean 1000 grain weight in gm.	Control	IAA 75	IAA 100	NAA 75	NAA 100 p.p.m.	
N ₁	34.2	35.7	35.4	37.7	35.3	35.7
N ₂	38.6	40.5	41.9	34.7	38.3	38.8
	36.4	38.1	38.7	36.2	36.8	

(Mean effects of nitrogen and interaction significant. S.D. (5 % P) for nitrogen, 2.1; for interaction, 4.7.)

Mean ear number per pot	Control	IAA 75	IAA 100	NAA 75	NAA 100 p.p.m.	
N ₁	27.0	26.4	25.0	22.6	24.8	25.1
N ₂	29.2	33.8	39.0	29.6	31.4	32.6
	28.1	30.1	32.0	26.1	28.1	

(Mean effect of nitrogen significant. S.D. (5 % P) for nitrogen, 2.8.)

¹ N₁, deficient in nitrogen; N₂, sufficient in nitrogen.

Table 6. *Effect of auxins and nitrogen on Punjab C 228 — 1953—54.*

Mean grain yield in gm per pot	Control	IAA 75	IAA 100	NAA 75	NAA 100 p.p.m.	
N ₁ ¹	29.9	29.9	33.0	33.8	42.3	33.8
N ₂ ¹	48.6	51.8	51.2	53.1	63.4	53.7
	39.2	40.9	42.1	43.5	52.9	

(Mean effects of nitrogen and auxin significant. S.D. (5 % P) for nitrogen, 5.9; for auxin, 9.2.)

Mean grain number per ear	Control	IAA 75	IAA 100	NAA 75	NAA 100 p.p.m.	
N ₁	28.8	32.4	32.5	29.8	30.9	30.8
N ₂	35.7	32.2	31.3	34.7	34.7	33.8
	32.3	32.3	31.9	32.3	32.8	

(Mean effect of nitrogen significant. S.D. (5 % P), 1.5.)

Mean ear number per pot	Control	IAA 75	IAA 100	NAA 75	NAA 100 p.p.m.	
N ₁	23.0	24.4	21.2	25.6	28.0	24.4
N ₂	30.4	33.6	32.2	33.4	38.6	33.7
	26.7	29.0	26.7	29.5	32.3	

(Mean effect of nitrogen significant. S.D. (5 % P), 4.2.)

Mean 1000 grain weight in gm.	Control	IAA 75	IAA 100	NAA 75	NAA 100 p.p.m.	
N ₁	42.2	37.8	46.5	45.9	48.4	44.2
N ₂	45.3	48.8	45.3	47.5	47.9	46.8
	43.8	43.3	45.9	46.7	48.3	

(Mean effects of nitrogen, auxins and interaction significant. S.D. (5 % P) for nitrogen, 1.8; for auxins, 4.1; for interaction, 4.3.)

¹ N₁, deficient in nitrogen; N₂, sufficient in nitrogen.

Table 7. *Effect of auxin and nitrogen in the field — 1953—54.*

Mean grain yield in lb per plot.			
	N.P. 710	C 228	
Control	9.4	11.5	10.45
NAA	10.3	10.9	10.60
Nitrogen 20 lb/acre	11.3	11.4	11.35
NAA+Nitrogen	11.3	12.0	11.65
	10.7	11.45	

the auxins generally increased the grain number at both levels of nitrogen. The interaction of nitrogen and auxins was also significant in the case of 1000-grain weight, the latter being differentially influenced by NAA and IAA at the two nitrogen levels. 100 p.p.m. NAA alone increased the grain yield of C 228 significantly due to its favourable effect on 1000-grain weight. This character was significantly favoured by NAA at the low level of nitrogen, there being little effect at the higher level.

Field Experiment of 1953—54

The interaction of nitrogen supply and auxin spray was studied on the varieties N.P. 710 and Punjab C 228. The experimental details are given below:

Layout: Simple randomization with four replicates.

Dimensions of single plot: 16' × 14' (16 rows, each 14' long, 200 plants per row).

Date of sowing: November 11, 1953.

Nitrogen application: Ammonium sulphate, 100 lb/acre on 11th January, 1954.

Spraying: NAA (75 p.p.m.) at the rate of 100 gallons/acre on December 9, 1953.

Weeding: Twice on December 9, 1953 and January 11, 1954 respectively.

The grain yield in lb per plot is given in table 7. Analysis of variance indicated that the mean grain yield of Punjab C 228 was superior to that of N.P. 710 and nitrogen increased the grain yield significantly. The effect of NAA on N.P. 710 was quite evident and in fact separate analysis of variance shewed it to be significant at 5 % probability.

Discussion

Sufficient experimental evidence has been presented to show that auxins like IAA and NAA stimulate the growth and yield of wheat. These auxins, when applied before the onset of the reproductive phase (as marked by the formation of double ridges), increased production as well as survival of tillers, number of leaves and spikelets on the shoot and even grain number and size. One may perhaps expect these auxins to stimulate tillering and differentiation of leaves and spikelets on the main shoot, as these processes continued actively during and soon after their application. Their subsequent effect on grain setting and filling is, however, worthy of note. A similar late effect on flower primordia of soaking seeds in auxin solution, combined with appropriate temperature, has been found by Leopold and Guernsey (1953).

The seasonal inconsistency of the effects of auxins is also worthy of note. We may consider the last two seasons in which some treatments were com-

mon. Variety C 228 consistently responded only to NAA in the two seasons, whereas N.P. 710 responded to NAA as well as IAA in the earlier season and to IAA only in the later. In 1952—53 NAA increased ear number in both the varieties, whereas in 1953—54 NAA and IAA increased grain number in N.P. 710 and NAA grain size in C 228. Inspection of the data on controls indicated that the 1953—54 season was more conducive to grain-filling. If so, it would follow that auxins only promote a trend in metabolism perhaps primarily imposed by weather conditions. Leopold and Guernsey (1953) have produced evidence on the interaction of auxin and temperature on the production of flower primordia in Wintex barley, teosinte, soybean, oats etc., (soaking of seeds only in auxin solution). They also found that the expression of the stimulative effect of auxins on flower number was modified by light intensity. Hussey and Gregory (1954) also did not find any stimulating effect of auxin on flower primordia of Wintex barley in two out of four experiments.

The promotive effect of auxins on spikelet differentiation, as observed earlier by Leopold and Thimann (1948), was confirmed in the 1952—53 experiment, there being an increase of 5 % only as against 40 % found by them and 10 to 15 % by Hussey and Gregory (1954). In the 1952—53 experiment, the spikelet number (mean of all ears) was not increased and also there was no effect on mean grain number. In the 1953—54 experiment, grain number per ear was increased by auxins, although the spikelet number was not; unfortunately the spikelets on the main axis were not counted separately.

Leopold and Thimann (1949) pointed out that the formation of vegetative buds was not promoted by auxins: in contrast, our data indicate that auxins increased spikelet as well as leaf number on the main axis.

TIBA also increased grain yield, ear number and leaf and spikelet number on the main axis in N.P. 710 in 1952—53. According to Leopold and Thimann (1949) agents like X-rays, TIBA and coumarin, which oppose the action of auxin, can promote the number of flower primordia in barley plants with a naturally high auxin content. The effect of TIBA on our plants cannot be interpreted on the assumption of naturally high auxin content, for, in that case NAA and IAA should have reduced the spikelet number on the main axis.

There was no evidence of interaction of nitrogen and auxin on the grain yield. Under pot-culture (1953—54), nitrogen and auxin increased yield more or less equally, whereas in the field the effect of the latter was much smaller. Despite this not very encouraging result and similar experience of other workers, it is felt that in view of the interaction of auxin with a number of internal and external factors, further investigation might yet reveal means for ensuring economic and consistent returns from its application.

Summary

The results of six experiments, carried out during five seasons, on the application of auxins (IAA and NAA) to wheat plants, through cut leaf tip or as spray, indicated that auxins increased production and survival of tillers, leaf and spikelet number on the main shoot, grain number and size and yield.

The effect of auxins on ear number and grain number and size varied with season, the three characters being not favoured together in the same season. Apparently weather conditions modified the response to auxins.

The increase in grain number per ear (mean of all ears) was independent of spikelet number. Auxins increased spikelet as well as leaf number on the main axis only.

Since auxins were applied just before the initiation of 'double ridges' on the main axis, it is noteworthy that their influence was exerted on late events such as setting and filling of grains.

There was no interaction of nitrogen and auxin on grain yield. In pot-culture, both increased yield equally but in the field the effect of the latter was much smaller. In view of the interference with the action of auxin by several factors, it is considered worthwhile to experiment further on the possibilities of its application.

The authors have the pleasure of thanking Dr. B. P. Pal, Director of the Institute, and Dr. S. M. Sikka, Head of the Division, for their interest and encouragement in the prosecution of this investigation.

References

- Cholodny N. G.: *Hormonisation of Grains*. — C. R. Acad. Sci. U.S.S.R., 3: 349. 1936.
Hussey G. & Gregory F. G.: *The Effect of Auxin on the Flowering Behaviour of Wintex Barley and Petkus Rye*. — Plant Physiol. 29: 292. 1954.
Kruyt W.: *A Study in Connection with the Problem of Hormonisation of Cereals*. — Acta Bot. Neerl. 3: 1. 1954.
Leopold A. C. & Thimann K. V.: *Auxin and Flower Initiation*. — Sci. 108: 664. 1948.
— *The Effect of Auxin on Flower Initiation*. — Amer. J. Bot. 36: 342. 1949.
— & Guernsey F. S.: *Modification of Flower Initiation with Auxins and Temperatures*. — Amer. J. Bot. 40: 603. 1953.
Pearse H. L.: *Growth Substances and their Practical Importance in Horticulture*. — Commonw. Bur. Horti. & Plantn. Crops. Tech. Comm. 20. 1948.

Proposed Molecular Structure for Straight and Branched Polymers of Glucose

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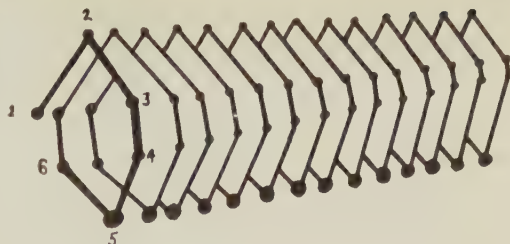
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Amylose has been described as a *linear* polysaccharide, the theory being that the glucose residues comprising it are linked in a long continuous chain of alpha 1 : 4 linkages. Studies have recently indicated that while the term »linear» may be applied to maltodextrins up to and including maltopentaose, to describe maltohexaose and polymers containing six or more glucose residues in alpha 1 : 4 linkages by the same term, may be erroneous (1) (2).

If the homologous series of glucose polymers: maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose and maltoheptaose, is subjected to partition chromatography, it is found that a regular, definite increment in R_f values exists from maltose through maltopentaose, but, that the R_f value for maltohexaose does not fall within the limits of this relationship (1) (3). Since there appears to be a direct relationship between the R_f value and the number of carbon atoms in a member of a homologous series (4), a difference in R_f value reflects differences of structure in a member. Then, obviously, maltopentaose and maltohexaose must differ in more than the presence of an extra glucose residue in maltohexaose, as is evident by quantitative analysis. A configurational difference between the two maltodextrins of this homologous series, must exist.

Further evidence of this structural difference is obtained by studies of the mode of action of branching enzyme. This enzyme adds glucose residues in an alpha 1 : 6 linkage to a polymer containing residues in alpha 1 : 4 linkages (1) (3) (5).

Figure 1. *Proposed structure for polyglucosides.* The helical molecule is shown in $3/4$ view. The glucose residues are numbered. The fifth glucose residue is exaggerated in each case to indicate its reactivity. Note that the front view is that of a hexagon (See text).



Branching enzyme branches maltohexaose, maltoheptaose, »linear« dextrans of 28 residues (6), 42 residues (7), and of course, amylose (8). However, maltopentaose and lower maltodextrins are not branched by the enzyme (1). In other words, there must be at least six glucose residues linked in an alpha 1 : 4 linkage for branching enzyme to utilize the dextrin for branching.

In the case of maltohexaose and maltoheptaose, the enzyme attaches an alpha 1 : 6-linked glucose residue only to the fifth residue of the chain (1). The indication is that the fifth residue in these two polymers is spatially different from the same residue in maltopentaose. From this evidence, the possibility suggested itself that the difference between maltopentaose and maltohexaose was one entirely of spatial arrangement. The configuration which fitted the experimental data best was that of a primary helix (1).

The figure shows a side view of such a structure. It will be seen that a front view of the structure would present a hexagonal pattern, not unsimilar to the ring configuration of glucose. There is much evidence for the presence of a six-unit basic structure, or multiple thereof (9) (10) (11).

It will be noted that all the apparently »reactive« molecules (the fifth molecules), in such a helical structure, would be in fairly close proximity to each other. This would be more compatible from the standpoint of enzyme action. In a chain-like straight polymer, the distance between each fifth residue would be much greater than in a helix.

The helical structure of amylose-iodine complex has been ventured to explain that color reaction (12) (13). Such a structure as basic for all polyglucosides has never been proposed, however.

In general, it can be stated that maltodextrins containing five or less glucose residues exist spatially, in a straight line, while those containing six or more residues tend to form a hexagonal-like helix. Maltopentaose, having only five glucose residues exists then, as a straight-chain polymer. The fifth residue is not reactive in this configuration, and hence, this polymer is not branched by branching enzyme.

Such a proposed structure as basic for all polyglucosides, brings up many interesting possibilities as far as enzyme action is involved.

For example, after exhaustive phosphorylase action on branched polysaccharides such as glycogen and amylopectin, a limit dextrin remains which contains six residues in an alpha 1 : 4 position and one residue in an alpha 1 : 6 position (14); the branch point is approximately at the fifth alpha 1 : 4 residue. Now, if the original »linear» polymer which was utilized by branching enzyme, were really a helix, then the severance of linkages at the same approximate points on the structure by the phosphorylase, would yield a simultaneous series of these same limit dextrans.

The author is indebted to J. Sheridan Mayer for his aid in construction of the figure showing the helix of polyglucosides, and to Dr. Richard Klein, Bronx Botanical Garden Laboratories, for his helpful criticism.

References

1. Fredrick, J. & Mulligan, F.: Mechanism of action of branching enzyme from *Oscillatoria* and the structure of branched dextrans. — *Physiol. Plant.* 8: 74. 1955.
2. — Structure of polysaccharides and the biochemical evolution of their synthesis in the algae. — *Biol. Rev. of City College* 17: 33. 1955.
3. Whelan, W., Bailey, J. & Roberts, P.: Mechanism of carbohydrase action. I. Preparation and properties of maltodextrin substrates. — *J. Chem. Soc.* 260: 1293. 1953.
4. Van Duin, H: Partition chromatography of homologous series. — *Biochim. et Biophys. Acta* 9: 580. 1952.
5. Fredrick, J.: Kinetics of polysaccharide formation in extracts of *Oscillatoria princeps*. — *Physiol. Plant.* 7: 182. 1954.
6. Peat, S., Whelan, W. & Bailey, J.: Enzymatic synthesis and degradation of starch. XVIII. Minimum chain length for Q-enzyme activity. — *J. Chem. Soc.* 292: 1422. 1953.
7. Nussenbaum, S. & Hassid, W.: Mechanism of amylopectin formation by the action of Q-enzyme. — *J. Biol. Chem.* 196: 785. 1952.
8. Bernfeld, P. & Meutemedian, A.: Isophosphorylase and the formation of branched chain polysaccharides. — *Nature* 162: 218. 1948.
9. Fredrick, J.: Branching characteristics of *Oscillatoria* polyglucosides. — *Physiol. Plant.* 6: 100. 1953.
10. Potter, A. & Hassid, W.: Starch. I. End group determination of amylose and amylopectin by periodate oxidation. — *J. Am. Chem. Soc.* 70: 3498. 1948.
11. Brown, F., Dunstan, S., Halsall, T., Hirst, E. & Jones, J.: Application of new method of end group determination to structural problems in the polysaccharides. — *Nature* 156: 785. 1945.
12. Hanes, C. & Cattle, M.: Relation of iodine color to structure; relation of color changes to development of reducing power during the hydrolysis of starch by different amylases. — *Proc. Roy. Soc. London B* 125: 387. 1938.
13. Swanson, M.: Iodine color of polysaccharides. — *J. Biol. Chem.* 172: 825. 1948.
14. Larnier, J., Illingworth, B., Cori, G. & Cori, C.: Structure of glycogens and amylopectins. II. Analysis by stepwise enzymatic degradation. — *J. Biol. Chem.* 199: 641. 1952.

The Apparent Necessity of Indoleacetic Acid for the Growth of *Diplodia* (Fungi Imperfecti)

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Introduction

It has now been established that indoleacetic acid (IAA) or compounds with similar biological activity are required by green plants for a number of morphogenetic and metabolic activities. IAA is actively associated with the growth of green flagellates (13), algae (7, 12), bryophytes (21, 29), pteridophytes (16, 28) and most of the seed plants. A requirement for IAA in normal growth behavior of non-chlorophyllous plants, however, has not been extensively demonstrated. Ball (3) and Beckwith and Geary (4) found that the growth of *Escherichia coli* and *Eberthella typhosa* was stimulated by low concentrations of IAA. Only four cases of positive effects of IAA on fungi have come to our attention. Anker (1) reported that the oxidation of glucose by *Saccharomyces cerevisiae* was accelerated in the presence of IAA; Fraser (14) found that it increased the growth rate of *Agaricus*; Nystrakis (23) demonstrated a large growth response of a strain of *Neurospora tetrasperma* to IAA; and Waygood, Mosie, and Kapica (26) recently reported that *Cladosporium herbarum*, grown on a pectin substrate, was stimulated by IAA, presumably by inducing the synthesis of pectin methyl esterase. For the most part, however, the addition of IAA or other growth substances to cultures of fungi failed to elicit any positive growth response and was usually inhibitory even in relatively low concentrations (9, 18, 24).

Despite these negative findings, certain reports strongly suggest that IAA may be of general importance in the economy of fungi. Its synthesis by fungi has been known for many years (5, 6, 22), and Dolk and Thimann (11) isolated and crystal-

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lized IAA from the culture liquids of *Rhizopus suinus* prior to its isolation from higher plants. If IAA were biologically inactive in fungi (27), enzymes necessary for its degradation would not be expected, but IAA-oxidase has been reported (25).

The consistent failure to obtain direct evidence for the metabolic participation of IAA led us to postulate that many fungi are normally autotrophic for this compound, synthesizing just those concentrations required for normal growth behavior. Under these conditions, experiments based on the hope that supplements of IAA might stimulate growth were unlikely to succeed since even small additions might be sufficient to raise the total IAA concentration to levels where toxic effects are evident. Very low concentrations of IAA would, similarly, be ineffective in inducing any measurable response. To establish an etiological role for IAA in the growth of fungi, a new experimental approach was needed and the experiments of McRae and Bonner (19), in which diortho substituted phenoxyacetic acids competitively and reversibly inhibited the action of IAA in *Avena* coleoptiles, provided such an approach. The present paper reports the results of growth studies on a strain of *Diplodia* in the presence of 2,4,6 trichlorophenoxyacetic acid (TCPA) or IAA and combinations of these two compounds. Following the rationale of Woolley (30) for elucidation of metabolic problems by the use of specific antimetabolites, the results reported here may be interpreted as providing presumptive evidence for a metabolic role for IAA in the growth of this fungus.

Materials and Methods

An isolate of *Diplodia*¹ (USDA strain 4725), obtained from Dr. G. B. Ramsey, U.S.D.A. Division of Handling, Transportation and Storage, Chicago, Illinois, was grown in Petri dishes on the following medium: asparagine, 2.0 gm; K_2HPO_4 , 1.0 gm; $MgSO_4 \cdot 7H_2O$, 1.0 gm; Hoagland's microelement supplement, 1.0 ml; glucose, 20 gm; agar, 20 gm; water to 1 liter, pH 7.0. Plugs were cut out with a cork borer (7 mm. dia.) from the advancing edge of the mycelium and were used to inoculate a liquid medium which had the same composition as above lacking agar only. All cultures were grown in 50 ml of this medium in 250 ml erlenmeyer flasks and were incubated at 26°C.

In every experiment, 5 replicates of each variable were set up. At the end of a 7 day growth period, mycelial mats were collected on filter paper and dried to constant weight at 80°C. IAA was determined by the procedure of Gordon and Weber (15); TCPA did not interfere with these analyses.

Analyses of variance of the data were carried out as described in Cochran and Cox (10) for a $4 \times 4 \times 5$ factorial experiment in completely randomized design. The least significant difference (LSD) between means was calculated by use of the *t* test, using the formula:

$$LSD = (t) s\bar{y}$$

¹ This isolate has been identified as *Diplodia natalensis* Pole-Evans. (Phytopath. 44: 471, 1954).

where:

$$s\bar{y} = \sqrt{\frac{2 s^2}{r}}$$

All conclusions are based on differences significant at the 1 % level.

Results and Discussion

Diplodia grew very uniformly in synthetic medium and synthesized IAA both in the presence and absence of tryptophane (Table 1). In the medium containing 5×10^{-3} M L-tryptophane the final IAA concentration reached 4×10^{-5} M in a week, a concentration which suppressed the growth about 25 per cent. Even in the absence of this IAA precursor, the final IAA concentration approached 2.8×10^{-6} M. It was observed that inhibitions of growth were apparent only after the fifth day of culture, at the time when the IAA increased to toxic levels.

If IAA, synthesized by the fungus in the presence of tryptophane, reaches a level which inhibits growth, addition of a specific antimetabolite should prevent this inhibition. Higher concentrations of the antimetabolite should be inhibitory. This line of reasoning has been used to advantage in studies on the IAA metabolism of roots by Burström (8). *Diplodia* cultures, grown in media with and without tryptophane, were supplemented with a wide range of concentrations of TCPA. In the presence of tryptophane (1×10^{-3} M), concentrations of 10^{-9} M and 10^{-10} M TCPA significantly increased the growth of the cultures (Figure 1). TCPA at 10^{-4} M and 10^{-5} M was, as expected, inhibitory, and intermediate concentrations were without significant effect on growth. When no IAA precursor was present (Table 2), TCPA did not significantly stimulate growth and concentrations as high as 10^{-6} M TCPA were significantly inhibitory. The failure of TCPA to stimulate growth suggests that in these cultures the level of IAA was optimal for growth. The significant inhibition of these cultures by a lower concentration of TCPA than that which inhibited the cultures supplemented with tryptophane tends

Table 1. *Effect of L-tryptophane on growth and IAA synthesis in Diplodia.*

	Tryptophane ($M \times 10^{-3}$)			
	0.0	1.0	2.5	5.0
Mg Dry Weight ¹	1132	970	945	850
Mg IAA/liter	0.48	3.00	4.34	7.00

¹ Dry weight of mycelia in 500 ml medium.

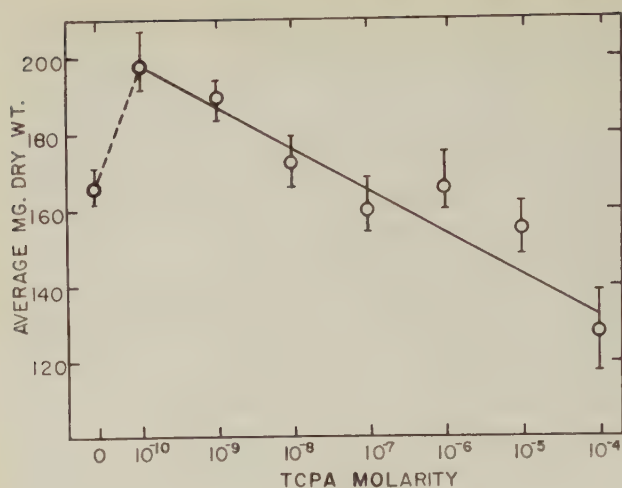


Figure 1. Effect of various concentrations of 2,4,6-trichlorophenoxyacetic acid on the growth of *Diplodia* grown in a medium containing 1×10^{-3} M L-tryptophane.

to strengthen this concept. Higher levels of TCPA apparently are required to affect growth when IAA is present in larger quantities. The absolute differences between the control cultures in each of the two series are of interest. Excessive levels of IAA derived from tryptophane suppressed the growth of the cultures independent of TCPA. This suppression could, however, be relieved by the addition of 10^{-10} M TCPA, a concentration which apparently was sufficient to balance out the excess IAA present in the cultures. These results demonstrate that TCPA is effective in preventing the deleterious effects of supraoptimal concentrations of IAA and also show that TCPA alone can inhibit the growth of *Diplodia*.

On the possibility that TCPA acted by suppressing the synthesis of IAA, analyses for IAA of culture filtrates containing TCPA were performed. No significant differences in IAA concentrations were found in the presence of all tested concentrations of TCPA indicating that the TCPA inhibitions of growth were not at the level of IAA biosynthesis.

Table 2. Effect of TCPA on growth of *Diplodia* cultures in the absence of tryptophane.

TCPA (M)	Dry Wt. (mg)	% Control
0	193	100
10^{-10}	201	104
10^{-9}	182	95
10^{-8}	183	95
10^{-7}	178	92
10^{-6}	158	82
10^{-5}	141	73
10^{-4}	139	72

Table 3. *Effect of IAA and TCPA on the growth of Diplodia*. Each figure represents the mean dry weight (mg.) of five cultures for each treatment.¹

TCPA ($M \times 10^{-5}$)	IAA ($M \times 10^{-5}$)			
	0.0	1.0	10.0	50.0
0.0	199.2	174.4	159.4	146.0
1.0	133.2	183.6	157.6	136.6
5.0	127.0	147.8	153.6	116.7
10.0	107.8	119.0	122.0	107.5

¹ LSD (1 %) = 15.1 mg.

To complete the demonstration that IAA is, in fact, required by *Diplodia* for normal growth behavior and that TCPA acts as a specific antimetabolite for the proper utilization of IAA, attempts were made to reverse the inhibitions induced by either of these compounds by the addition of the other. The analysis of experiments of this type, in which both metabolite and antimetabolite are independently toxic, is more difficult than for those in which growth can be made dependent on the exogenous supply of the metabolite such as IAA for cell elongation of *Avena* coleoptiles and the need for succinate in cytochrome c oxidations *in vitro*. To demonstrate a metabolite—antimetabolite relationship with IAA and TCPA, one should be able to show not only that the addition of the metabolite can reverse the growth inhibitions induced by the antimetabolite, but also that the inhibitions induced by the normal compound can be reversed by the antimetabolite. According to Audus (2), such data are not amenable to kinetic analysis of the classic Lineweaver-Burk type as applied by McRae and Bonner (20) to the TCPA—IAA interaction in tissues which do not synthesize IAA.

Metabolite—antimetabolite interaction experiments were set up using both control medium and medium supplemented with 1×10^{-3} M tryptophane. Three concentrations each of IAA and TCPA, determined by preliminary experiments to give good inhibitions, were used in all combinations. In the tryptophane series, the concentrations of TCPA were increased so as to obtain antimetabolite levels high enough to induce significant inhibition.

The control cultures grown in the normal and tryptophane-supplemented media averaged 199.2 mg dry weight (Table 3) and 125.6 mg dry weight (Table 4), respectively. Again, the presence of excess IAA arising from tryptophane was sufficient to depress the growth of the fungus. Several points are immediately apparent. IAA inhibited the growth of *Diplodia*, the absolute inhibition being greater in the presence than in the absence of tryptophane. This finding is in agreement with those of other workers that supplements of IAA are toxic. TCPA is also independently inhibitory.

Table 4. *Effect of IAA and TCPA on the growth of Diplodia.* Medium contained 1×10^{-3} M L-tryptophane. Each figure represents the mean dry weight (mg.) of five cultures for each treatment.¹

TCPA ($M \times 10^{-5}$)	IAA ($M \times 10^{-5}$)			
	0.0	1.0	10.0	50.0
0.0	125.6	108.0	82.3	74.0
5.0	103.8	121.0	92.4	91.2
10.0	70.2	83.2	72.0	68.6
50.0	50.2	52.2	107.0	94.6

¹ LSD (1%) = 27.8 mg.

In the series of cultures grown without added tryptophane (Table 3), 1.0 and 10.0×10^{-5} M IAA reversed the inhibitions induced by 1.0 and 5.0×10^{-5} M TCPA. At the lower concentration of TCPA, 1.0×10^{-5} M IAA was more effective in reversing the inhibition than 10.0×10^{-5} M IAA. At the higher concentration of TCPA, the reverse was true. Apparently the addition of IAA above the quantity necessary to reverse the TCPA inhibition results in a toxic effect. The inhibition of growth induced by 10.0×10^{-5} M TCPA could not be reversed with the concentrations of IAA used. Also, growth inhibition induced by IAA could in no case be reversed by TCPA in our experiments.

There were significant changes in the series in which the medium contained 10^{-3} M tryptophane (Table 4). A concentration of 5×10^{-5} M TCPA, which was previously severely inhibitory, failed to inhibit growth, presumably due to the increased quantity of IAA present in the medium from the conversion of tryptophane. For the same reason, the same concentrations of IAA as used in the previous series had a more marked inhibitory effect. In this series, the inhibition induced by 50.0×10^{-5} M TCPA was reversed by 10.0 and 50.0×10^{-5} M IAA. Again the inhibitions induced by IAA could not be reversed by TCPA.

The data presented here show that IAA and TCPA are independently inhibitory in the growth of *Diplodia*. The former finding may be explained by the assumption that the exogenous supply of IAA was sufficient to raise the level of this compound to a toxic value. This assumption is in accord with the hypothesis of a two-point attachment of the IAA molecule to a receptor site and the suggestion that an antiauxin acts by blocking the complete attachment of the IAA to these sites (20). McRae and Bonner (20) and Ingestad (17) have developed complete mathematical expressions of these hypotheses.

We have shown that the inhibition induced by one substance (TCPA) could be reversed by another substance (IAA) which is itself independently inhibitory. On the other hand, the inhibition induced by IAA could not be reversed

by TCPA in the concentrations used. If IAA and TCPA are competing for a single locus required for growth (whether the locus possesses one, two or more obligatory sites of attachment for activity), and the relative affinities of the IAA and TCPA molecules for this locus are different, i.e., K_m of IAA > K_m of TCPA (20), it is manifestly impossible for TCPA, in concentrations less than those of IAA, to prevent an inhibition of growth by an excess of IAA.

On the basis of the work of McRae and Bonner (20), it is assumed that the affinity of IAA for the «sites of activity» on their hypothetical growth enzyme is much greater (1—2 orders of magnitude) than the affinity of TCPA for the same sites. Thus, IAA would tend to remain on these sites until the concentration of TCPA was overwhelming (at least 50—100 times as great as IAA). The chance of achieving this situation experimentally is low, particularly since the solubilities of the substances differ and we know nothing of the relative concentrations of the materials at the active site. On the other hand, the reversal of the TCPA inhibition should, and did, occur in our experiments, since IAA will tend to replace TCPA at these sites due to its relatively greater affinity. Here then is a reason for the failure to reverse the IAA inhibition. However, when a range of TCPA concentrations was tested in the presence of tryptophane, the tryptophane inhibition (as compared with control medium) was relieved. In this instance one can assume that the tryptophane inhibition was due to excess IAA synthesized within the cell at or near the loci of attachment of the newly formed IAA.

While these experiments do not invalidate the hypotheses of McRae and Bonner, they cannot be said to support them. Whether they can be rationalized into a scheme in which a «growth enzyme» with IAA as a cofactor is the key is doubtful (2, 17). Only additional studies on the problem, with special attention paid to the relative penetrations of these materials into the cell and the nature of the locus of attachment, can answer these questions.

Summary

Cultures of *Diplodia* were grown in synthetic liquid media containing various concentrations of indoleacetic acid and an antimetabolite for IAA, 2,4,6 trichlorophenoxyacetic acid. Each of these substances was independently inhibitory for growth but, in certain combinations, the inhibitory effects of TCPA were reversed partially or wholly by IAA.

Our results indicate that IAA, for which this fungus is autotrophic, presumably is required for growth and is unlikely to be a functionally neutral, metabolic end-product. The results show that the endogenous IAA concentrations in the cultures are close to those required for optimal growth and that

their biosynthesis is carefully regulated. The TCPA does not interfere with growth by affecting the biosynthesis of IAA but may act as an inhibitor of its action. The implications of these findings are discussed.

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References

1. Anker, L.: Influence of indole-3-acetic acid on the respiration of *Saccharomyces cerevisiae*. — *Proc. Koninkl. Neder. Akad. Wet.* 52: 875. 1949.
2. Audus, L. J.: Auxin antagonists and synergists. — *New Phytol.* 53: 461. 1954.
3. Ball, E.: Heteroauxin and the growth of *Escherichia coli*. — *J. Bact.* 36: 559. 1938.
4. Beckwith, T. D., & Geary, E. M.: — Effect of indol 3 acetic acid on the multiplication of *Esch. coli* and *E. typhosa*. — *J. Infect. Diseases* 66: 78. 1940.
5. Bonner, J.: The production of growth substances by *Rhizopus suinus*. — *Biol. Zentbl.* 52: 565. 1932.
6. Boysen-Jensen, P.: Über die Bildung und biologische Bedeutung des Wachstums-Regulators bei *Aspergillus niger*. — *Biochem. Zeit.* 250: 270. 1932.
7. Brannon, M. A. & Sell, H. M.: Effect of 3-indoleacetic acid on the dry weight of *Chlorella pyrenoidosa*. — *Am. J. Bot.* 32: 257. 1945.
8. Burström, H.: Studies on growth and metabolism of roots. IV. Positive and negative auxin effects on cell elongation. — *Physiol. Plant.* 3: 277. 1950.
9. Bünning, E.: Wachstum und Stickstoffassimilation bei *Aspergillus niger* unter dem Einfluss von Wachstumsregulatoren und von Vitamin B. — *Ber. Deutsch. Bot. Ges.* 52: 432. 1934.
10. Cochran, W. G. & Cox, G. M.: *Experimental Designs*. — John Wiley and Sons, N. Y. 1950.
11. Dolk, H. E. & Thimann, K. V.: Studies on the growth hormone of plants. — I. *Proc. Nat. Acad. Sci. (U. S.)* 18: 30. 1932.
12. DuBuy, H. G. & Olson, R. A.: The presence of growth regulators during the early development of *Fucus*. — *Am. J. Bot.* 24: 609. 1937.
13. Elliot, A. M.: The influence of certain plant hormones on the growth of protozoa. — *Physiol. Zool.* 11: 31. 1938.
14. Fraser, I. M.: Effects of indoleacetic acid on growth of *Agaricus*. — *Aust. J. Biol. Sci.* 6: 379. 1953.
15. Gordon, S. A. and Weber, R. P.: Colorimetric estimation of indoleacetic acid. — *Pl. Physiol.* 26: 192. 1951.
16. Hurel-Py, G.: De l'influence de l'acide indole-3-acétique sur la culture des prothalles d'*Asplenium* en milieu aseptique. — *C. R. Soc. Biol.* 137: 57. 1943.
17. Ingestad, T.: Kinetic aspects on the growth-regulating effect of some phenoxy acids. — *Physiol. Plant.* 6: 796. 1953.
18. Leonian, L. H. & Lilly, V. G.: Is heteroauxin a growth-promoting substance? — *Am. J. Bot.* 24: 135. 1937.

19. McRae, D. H. & Bonner, J.: Diortho substituted phenoxyacetic acids as antiauxins. — Pl. Physiol. 27: 834. 1952.
20. — Chemical structure and antiauxin activity. — Physiol. Plant. 6: 485. 1953.
21. Moewus, F. & Schader, E.: Über den Einfluss von Wuchs- und Hemmstoffen auf das Rhizoidwachstum von *Marchantia* Brutkörpern. — Beitr. Biol. Pflanzen 29: 171. 1952.
22. Nielsen, N.: Untersuchungen über einen neuen wachstumsregulierenden Stoff: Rhizopin. — Jahrb. Wiss. Bot. 73: 125. 1930.
23. Nyterakis, R.: Resistance de *Neurospora tetrasperma* aux fortes doses d'auxine et relation avec l'hypothèse de l'existence des »metabolites antiauxines». — C. R. Acad. Sci. 238: 143. 1954.
24. Richards, R. R.: Responses of representative fungi to certain growth-regulating substances. — Bot. Gaz. 110: 523. 1949.
25. Sequeira, L. and Steeves, T. A.: Auxin inactivation and its relation to leaf drop caused by the fungus *Omphalia flava*. — Pl. Physiol. 29: 11. 1954.
26. Waygood, E. R., Mosie, J., and Kapica, L.: The effect of plant growth regulating substances on the production of pectin esterase by *Cladosporium herbarum* (Pers.) Link. — Paper presented at the 29th meeting of the Amer. Soc. Plant Physiol. Sept. 1954.
27. Went, F. W. & Thimann, K. V.: Phytohormones. — Macmillan and Co. N. Y. 1937.
28. Williams, S.: Correlation phenomena and hormones in *Selaginella*. — Nature 139: 966. 1937.
29. v. Witch, H.: Versuche über den Einfluss von Wuchsstoffe auf Wachstum und Entwicklung von *Calypogeia trichomanes*. — Planta 30: 664. 1940.
30. Woolley, D. W.: A Study of Antimetabolites. — John Wiley and Sons. N. Y. 1952.

Growth Studies in Woody Species VII. Photoperiodic Control of Germination in *Betula pubescens* Ehrh.

By

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It is well-known that the flowering response of both 'short-day' and 'long-day' herbaceous species is governed by the day-length conditions to which the *mature leaves* are exposed, and that the meristematic tissue of the shoot apex is insensitive. It has been shown for certain woody species, however, that direct photoperiodic perception may occur in the meristematic tissue of both dormant resting-buds and actively-growing apices, in relation to dormancy phenomena. Thus, the unchilled resting-buds of dormant, leafless seedlings of various woody species may be induced to expand by exposure to long-day conditions (Olmsted, 1951; van der Veen, 1951; Wareing, 1953). Similarly, actively-growing, *leafy* seedlings of *Betula pubescens* may be induced to form resting-buds by direct exposure of the apical region of the shoots to short-day conditions, regardless of the conditions of day-length under which the mature leaves are maintained (Wareing, 1954). These observations suggested that possibly the embryos of such woody species might show photoperiodic effects in relation to dormancy, and experiments were carried out to investigate this possibility. Seeds of birch (*Betula pubescens*) were selected for this purpose, since they are small and have a relatively translucent pericarp, so that a high proportion of the incident light must reach the embryo. A preliminary report of this investigation has already been published (Black and Wareing, 1954).

Experimental

General technique

The birch seeds used were derived from open-pollinated trees growing in Southern England (supplied by courtesy of the British Forestry Commission) and were stored at room temperature.

To test the effect of a particular treatment, seeds were sown on filter paper moistened with distilled water, and the germination percentage was determined at the end of the experimental treatment. Since a large proportion of the seeds do not contain embryos, there were no advantages in planting a constant number for each treatment. For this reason, and since the seeds are quite small (about 2 mms. long), approximately 300 were planted in each dish, and at the end of the treatment the number of fertile seeds which had not germinated were determined by dissection. For most treatments the total number of fertile seeds was found to be approximately 100, but in no case was this number less than 80.

Most of the experiments were carried out using 'monochromatic' red light falling within the range 5800 Å—7000 Å, obtained from red fluorescent tubes in conjunction with red «Perspex» filters (R. 400). In all experiments, except when stated otherwise, the intensity at the level of the seeds was approximately 1000 ergs/cm²/sec. Two cabinets containing these tubes were specially constructed. These were situated in a cold room (temperature 5°C) and the temperature in each cabinet was thermostatically controlled at 15° or 20°C.

Infra-red light was obtained from a 160-watt tungsten filament lamp with an infra-red gelatine filter ('Ilford' No. 207) transmitting the wavelengths from 7300 Å. The filters were used in conjunction with water screens of approximately 9.5 cms. thickness which effectively cut off the radiation beyond 10,000 Å.

In the case of the red light the intensity was measured by a barrier-layer photocell which had been calibrated in absolute energy units against a thermopile. The infra-red radiation was measured by means of a photocell (G.E.C. type C.M.V. 6) having a caesium-silver oxide cathode. The initial calibration of both photocells was carried out for the particular light sources used in the experiment.

The Influence of Daylength

Preliminary tests showed that the *unchilled* seeds will not germinate on moist filter-paper in complete darkness at room temperature, but will do so if exposed to continuous illumination at 45 foot-candles from a 100-watt tungsten filament lamp. They are thus light-requiring seeds. The following experiments were then carried out to determine the effect on germination of exposing the seeds to various lengths of photoperiod.

The experiments were carried out in the growth cabinets at a controlled temperature of 15°C, using red light at 1000 ergs/cm²/sec. After the exposure to the required daily photoperiod the seeds were placed in the dark in light-proof boxes, under the same temperature conditions, for the remainder of the 24-hour cycle.

Eight different durations of photoperiod were used, namely, 2, 4, 8, 12, 16 and 20 hours. In addition, one dish of seeds was kept in darkness throughout, and another

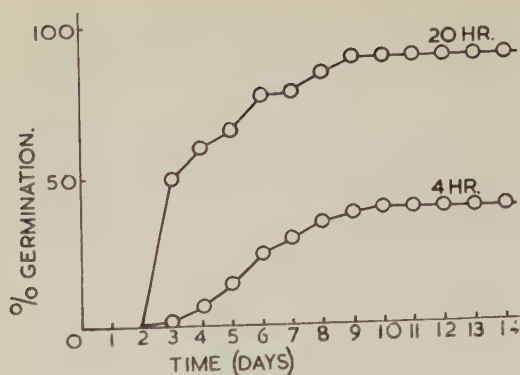


Figure 1. Daily germination of seeds under long-day and short-day conditions.

subjected to continuous illumination. The final percentage germination was determined only when there was no further increase in the number of germinated seeds. This was after 14 days. (See, for example, Figure 1).

The results of the treatments are shown in Figure 2. The difference in response of unchilled seed to 'long'- and 'short days' has been confirmed many times, from observations on 'controls' used in other experiments. In all cases, a low germination figure (of the order of 30 per cent) results when seeds are given 'short days' and a higher percentage germination is obtained under 'long days'.

It can be seen from Figure 2 that there is no 'critical' daylength for the breaking of dormancy of these seeds. As the length of the photoperiod is increased there is a corresponding increase in the percentage of seeds which germinate. However, there is nothing to suggest that the total duration of illumination to which the seeds are exposed is the factor controlling the final germination percentage, for in the case of the seed exposed to short photoperiods no further germination is obtained after 10 days, even if the treatment is continued. (Figure 1). Thus it is apparent that the total light-exposure is not the factor which brings about the germination of birch seeds. Further experiments (pp. 304—6) have confirmed this conclusion.

Effect of temperature on photoperiodic response

In the course of one experiment it was found that the 'control' seeds exposed to 8-hour photoperiods gave a much higher germination than expected, of the order of 90 per cent. On investigation it was decided that the only new factor, compared with those experiments which gave low percentages with short days, was the temperature, which was higher (approximately 20 °C) than in earlier experiments. Further experiments were therefore

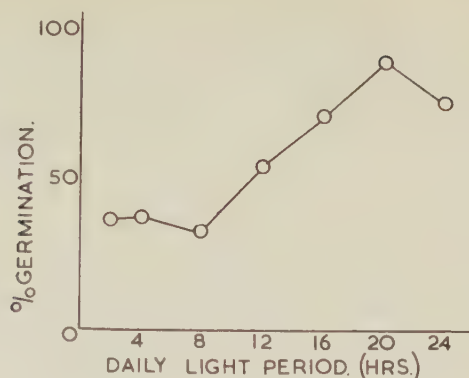


Figure 2. *Effect of day-length on germination.*

designed, with the purpose of determining what influence the temperature has on photoperiodic response.

Four batches of unchilled seeds were sown. At controlled temperatures of 15° C and 20° C, seeds were exposed to 8-hour and 20-hour daily photoperiods, respectively. The germination obtained from each treatment is shown in Table 1.

It is seen that a 'photoperiodic' response is obtained only at the lower temperature. At the higher temperature a high percentage germination is obtained with both long and short photoperiods.

Radiation-intensity requirements

The following experiment was carried out to determine the radiation intensities required for the photoperiodic promotion of germination at 15° C. Seeds were maintained under 8-hour and 20-hour daily photoperiods at three different intensities viz. 250, 700 and 1400 ergs/cm²/sec. Each treatment was continued for 10 cycles at 15° C, after which the seeds were stored in the dark at the same temperature. The results are shown in Table 2. Germination percentages of the same order were obtained with all of the three intensities used. It is thus evident that an intensity of even 250 ergs/cm²/sec. is above the 'saturation' value, and that with the intensity of 1000 ergs/cm²/sec. used

Table 1. *The effect of temperature on photoperiodic response.*

Photoperiod	15° C	20° C
8 hours	34.3 %	93.2 %
20 hours	90.0 %	93.0 %

Table 2. *Effect of radiation-intensity on germination. Temperature: 15° C.*

Intensity (ergs/cm ² /sec)	8 hour photoperiods	20 hour photoperiods
250	23.3 %	85.0 %
700	26.4 %	85.2 %
1400	26.1 %	72.7 %

in most of the experiments, the total quantity of light (time×intensity) was not the factor limiting germination under short day conditions.

In further experiments, carried out with single light-exposures at 20° C, it was found that an intensity of 60 ergs/cm²/sec. may produce as high a germination percentage as one of 2500 ergs/cm²/sec., provided that the light-exposure exceeds 1 hour's duration.

Summation effects

The following experiment was carried out to determine the number of cycles required to give maximum germination under both long and short photoperiods.

Seeds were exposed to a number of daily 8-hour and 20-hour photoperiods at 15° C after which they were stored in the dark at 15° C. With both the long and the short photoperiods a number of cycles from 1—10 were used. After storage in the dark for 12 days the germination percentages were determined and are shown in Table 3.

It is seen that eight cycles are sufficient to give the maximum germination percentage under both long and short days.

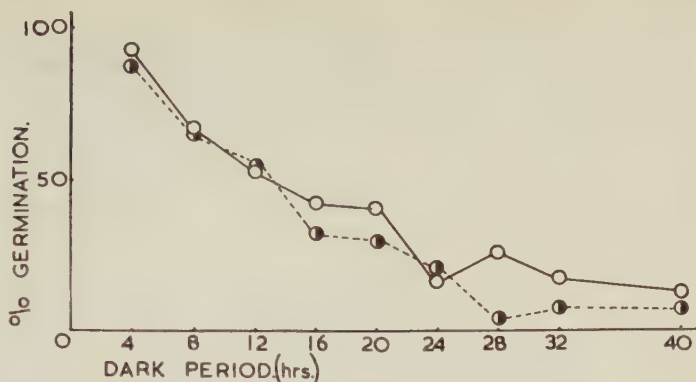
Relative roles of the light and dark periods in the photoperiodic promotion of germination.

In the case of herbaceous species it has been established that the flowering response of both 'long-day' and 'short-day' species is determined by the absolute lengths of the dark period and, also, in some species, of the light

Table 3. *Percentage germination resulting from varying number of cycles.*

Duration of photoperiod	Number of cycles								
	2	3	4	5	6	7	8	9	10
8 hours.....	—	0	8.9	15.4	19.0	27.5	33.0	33.7	32.0
20 hours.....	2.0	18.8	20.0	40.2	55.3	80.5	87.6	89.1	88.6

Figure 3. Effect of varying durations of dark period, with constant daily photoperiods of 20 hours (—○—) or 8 hours (—●—).



period, e.g. Biloxi soybean (Hamner, 1940). Similarly, in the photoperiodic control of seed germination the problem which arises is whether, with 'short-days', low germination percentages are obtained because the light period is too short or because the dark period is excessively long. Similarly, under 'long-day' conditions the greater germination may be determined either by the length of the photoperiod or by the shortness of the dark periods. The experiments described below were designed to investigate these problems.

Experiment 1. In this experiment constant light periods of 8 hours and 20 hours respectively, were used in conjunction with various lengths of dark period, so that 9 different treatments were given for each photoperiod, at a temperature of 15° C. Eight cycles of light and dark were used for each treatment, so that for each duration of photoperiod the total light exposure was the same. Following the eight cycles of treatment, the seeds were stored in the dark at 15° C for two weeks, after which time the germination percentages were obtained. These are presented in Figure 3.

This experiment clearly shows that the absolute length of the dark period plays a determinative role, for with both 8-hour and 20-hour photoperiods there is a marked reduction in the percentage germination as the dark period is lengthened. Moreover, high germination percentages are obtained even with 8-hour photoperiods, provided that the associated dark periods are short.

Experiment 2. The effects of exposing seeds to different lengths of photoperiod using dark periods of constant duration were studied in the following experiment. In the first part of the experiment a dark period of 4 hours was used in conjunction with different lengths of photoperiod, so that 7 treatments were obtained. After exposure to 9 inductive cycles the seeds were placed in dark storage. The temperature throughout the experiment was 15° C. In the second part of the experiment a 20-hour dark period was associated with the photoperiods. In all other respects the treatment was the same as

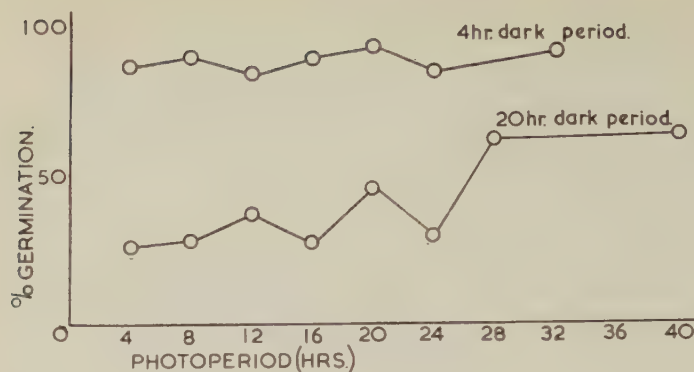


Figure 4. *Effect of varying durations of photoperiod, with constant dark periods of 4 hours or 20 hours.*

with the 4 hour dark period. The results of this experiment are given in Figure 4.

As would be expected from a consideration of the results of Experiment 1, seeds which are maintained in short dark periods, no matter what the length of the accompanying photoperiods, show high germination percentages. This is further evidence for the determinative role of the absolute length of the dark period. With a 20-hour dark period there is little germination when this is given in conjunction with short light periods. An increase in the length of the photoperiod, however, brings about a corresponding increase in the number of seeds which germinate.

Effect of a 'light-break' given during the dark period

It is well-known that in herbaceous species the flowering response is affected by the absolute length of the dark period, and that this response is markedly modified if the dark period is interrupted with a short period of illumination. It is therefore of considerable interest to investigate the effect of a 'light-break' during the dark period, on seed germination. The groups of seeds were exposed to the following light regimes at 15° C: —

- a) 6 hours light, alternating with 6 hours dark.
- b) 12 hours light, alternating with 12 hours dark.
- c) 12 hours light, alternating with 12 hours dark, with a 1-hour light break at full intensity (1000 ergs/cm²/sec) in the middle of the dark period.

The exposures were continued for 9 days, after which time the seeds were maintained in the dark at the same temperature. The results obtained after 12 days storage, are shown in Table 4.

The light-break clearly has a stimulatory effect on germination.

Table 4. *The effect of a 'light-break' on germination. Temperature: 15° C.*

Treatment		Percentage germination
Light period	Dark period	
6 hours	6 hours	86.9
12 hours	12 hours	49.0
12 hours	12 hours (with 1 hour light break)	82.7

Effects of increased temperature

It has been shown for both long-day and short-day herbaceous species that an increase in temperature during the dark period tends to enhance the effectiveness of the latter. This is to be expected, of course, if the dark reactions have a positive temperature-coefficient. The reverse seems to be true in the photoperiodic responses of birch seed, where the 'short-day' effects are lost when the temperature is increased to 20° C. An attempt was made to obtain more information on these temperature effects, in relation to both the light and dark periods, by using single light periods and varying the temperature during both the photoperiod and the following dark storage period.

Experiment 1. Seeds were exposed to single photoperiods at 15° C and 20° C respectively and then stored in the dark at the same temperatures. Preliminary tests demonstrated that seeds were not responsive to light after only 15 minutes imbibition. In all experiments, therefore, the seeds were allowed to imbibe water in the dark for one hour previous to the illumination. The seeds were watered as necessary during the two weeks' storage in the dark. The results are shown in Table 5.

Similar results were obtained in a repeat of this experiment. It is seen that, at 15° C, exposure to a single photoperiod, even of 24 hours' duration, results in only a low percentage germination. A small rise in temperature to 20° C, on the other hand, greatly increases the response to a single photoperiod. The processes involved in germination thus appear to have a very high overall temperature coefficient.

Table 5. *Percentage germination following single light exposures, at two different temperatures.*

Temperature (in both light and dark)	Duration of light period								
	2 mins.	15 mins.	1 hr.	4 hrs.	8 hrs.	12 hrs.	16 hrs.	20 hrs.	24 hrs.
15° C	—	0	4.0	3.9	8.5	—	4.3	5.0	1.6
20° C	17.5	31.0	—	55.3	74.3	90.8	88.6	89.6	92.8

Table 6. *Percentage germination following single light exposures, at different temperatures during light and dark. Temperature: light 15° C; dark 20° C.*

Duration of light exposure	2 mins.	15 mins.	4 hrs.	8 hrs.	12 hrs.	16 hrs.	20 hrs.	24 hrs.
Percentage germination ...	27.0	52.4	61.5	62.9	65.3	69.0	72.4	71.5

Experiment 2. It is not possible, from the results of the preceding experiment, to determine whether it is the light reaction or the following dark reactions which are primarily affected by an increase in temperature from 15° C to 20° C. In the following experiment the same photoperiods were used as in Experiment 1, at a temperature of 15° C. The temperature during the dark storage period was maintained at 20° C.

It is known that there is a relationship between the sensitivity of seeds to light and the length of time for which these have been imbibing water (Evenari & Neumann, 1953.) Borthwick and his co-workers (1954) have recently investigated this relationship, and have found that there is a gradual increase in sensitivity to light with time, the maximum being attained after 12—20 hours imbibition. In the present experiment seeds were therefore allowed to imbibe water for some time previous to exposure. Thus those seeds which were to be exposed for 2 minutes and 15 minutes respectively were allowed to soak in the dark for nearly 20 hours before illumination. For the longer photoperiods it was arranged that the middle of the light period occurred 20 hours after the commencement of soaking.

The results are shown in Table 6. The increased germination at 20° C, observed in the previous experiment, is still obtained if the higher temperature treatment is restricted to the period of dark storage following a single light exposure. These results were confirmed in a repeat experiment.

Although the increased germination obtained on raising the temperature from 15° C to 20° C is thus not primarily due to an effect upon the photo-reaction, nevertheless the increased temperature must affect certain 'dark' reactions initiated by the light-exposure, as is shown by the following experiment.

Two dishes of seeds were exposed to light for 24 hours at 15°C. One dish was then placed in the dark at 20°C for 4 days and then transferred to the dark at a temperature of 15°C. The second dish of seeds was maintained in the dark at 15°C throughout. A third group of seeds was kept in the dark at 20°C for 4 days prior to receiving the 24-hour light-exposure. This group was then placed in the dark at 15° C. For seeds kept at the higher temperature *after* the photoperiod a germination figure of 74.3 per cent was obtained, as opposed to 10.9 per cent for seeds given the higher temperature treatment *before* the light-exposure. Those seeds maintained at 15°C throughout gave 5.4 per cent germination.

Thus it is clear that, for the higher temperature to be effective, it must be given *after* the light exposure, and it therefore appears that it is the reactions initiated by red light which are promoted at the higher temperature.

Table 7. *Effect of various temperatures during the light and dark periods.*

12-hour photoperiod	12-hour dark period	Percentage germination
15° C	15° C	54.5
15° C	20° C	97.0
20° C	15° C	94.5
5° C	20° C	93.7

Although, in experiments with a single light exposure, it is the temperature during the period of dark storage which is primarily effective in stimulating germination, nevertheless an appreciable further increase in germination is obtained when the temperature during the light period is also 20° C (Compare Tables 5 and 6). This conclusion is directly confirmed by the results of the following experiment, in which a series of photoperiodic cycles were used.

Experiment 3. Four groups of seeds were maintained in 12-hour daily photoperiods, in conjunction with various temperatures during the light and dark periods as shown in Table 7. It is clear from the results that with repeated cycles, high temperature in either the dark or the light period is capable of stimulating germination. Even with the low temperature of 5° C. during the photoperiod, a high germination is obtained if the temperature during the associated dark period is 20° C.

Experiment 4. The foregoing experiments demonstrate that a higher temperature in the dark storage period following a single photoperiod serves to increase the amount of germination. The present experiment was designed to give further information on the temperature effect by determining whether the higher temperature, to be effective, is necessary *immediately* after the light period, or at any time during the period of dark storage.

Twenty-four groups of seeds were sown. Twelve groups were illuminated for 4 hours at 15°C, and the remainder exposed to light for 24 hours at the same temperature. After the exposure all the seeds were placed in dark storage. The dark storage period was divided into two temperature regimes, so that immediately after the light exposure 6 dishes of seeds which had been exposed to light for 4 hours and 6 which had been illuminated for 24 hours were placed in the dark at 20° C, and the remaining 6 from each exposure, at 15°C. After a certain number of days each dish of seeds was transferred (in the dark) to the lower or the higher temperature. The duration of the dark storage period was 10 days, so that the following treatments were given to seeds exposed to both photoperiods: —

- a) 1—5 days at 15°C followed by 9—5 days at 20°C.
- b) 1—5 days at 20°C followed by 9—5 days at 15°C.
- c) 15°C throughout, and 20°C throughout (for 10 days).

This experiment was repeated several times, and although in some repeats there was a small amount of variation, similar results were obtained in all cases.

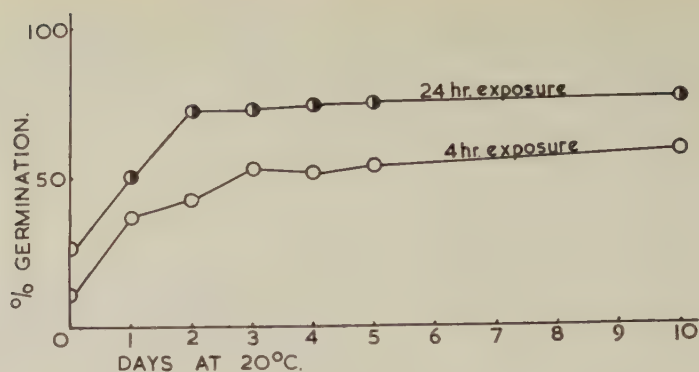


Figure 5 a.

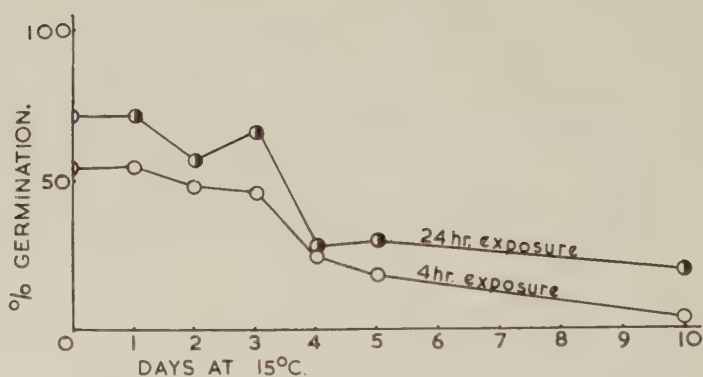


Figure 5 b.

Figure 5. *Effect of temperature during dark storage, following single light-exposure for 4 hours or 24 hours. (a) Seeds stored in dark at 20° C for given number of days and then transferred to 15° C. (b) Seeds stored in dark at 15° C for given number of days and then transferred to 20° C.*

The results of these treatments are shown in Figure 5. In the case of the seeds exposed to light for 24 hours it is apparent that in order to obtain a high percentage germination they must be maintained at the higher temperature for at least two days immediately after illumination. A longer time at 20° C does not significantly increase the amount of germination, but with less than two days lower percentages are obtained. Similarly, with the seeds exposed to the 4-hour light period the highest percentages of germination are obtained for all seeds kept at 20° C for at least 3 days immediately following the exposure to the photoperiod. Low germination is obtained with seeds which have been stored at 15° C for several days before transfer to 20° C. Those seeds which had been kept at 15° C for only one day prior to removal

Table 8. *Percentage germination with infra-red radiation given at various intervals following exposure to red light.*

Infra-red exposure given at following intervals after exposure to red light (hours):												
0	3	5	6	7	8	10	12	14	20	24	46	72
0	0	3.3	13.9	22.2	26.3	29.4	42.0	47.5	44.0	47.9	53.1	51.5

Control (no infra-red): 49.0 per cent.

to the higher temperature conditions gave as high a germination percentage at those which were at 20° C throughout. As the number of days of storage at 15° C increases, there is a corresponding decrease in the number of seeds which subsequently germinated. It is thus apparent that those reactions which are promoted at the higher temperature are completed in about 2 days. Moreover, if the high temperature requirements are not fulfilled immediately after exposure to light there appears to be a decay of the capacity to respond to the higher temperature.

Effect of infra-red radiation on germination

It has recently been shown that the photoreaction promoting germination in lettuce seeds is reversible by infra-red radiation (Borthwick et al 1952, 1954). Experiments described below have shown that the same phenomenon occurs in birch seeds.

Experiment 1. Two groups of seeds were exposed to red light for 2 hours at 15° C and then placed in the dark at 20° C. One group was irradiated with infra-red at an intensity of 3000 ergs/cm²/sec. for 30 minutes before the dark storage. During this treatment the glass top of the petri dish was removed so that the seeds were directly exposed to the radiation. Similar treatment was given to two other groups of seeds which had been exposed to 4 hours of light. After storage in the dark for two weeks the germination percentages were determined.

With the exposures to red light only, a germination of approximately 34 % was given with both 2 and 4 hours' illumination, but with infra-red radiation following the red light, the germination in both cases was nil. It is clear from these results that exposure to infra-red radiation nullifies the effect of previous exposure to red light, as in lettuce seed.

Experiment 2. An experiment was carried out to determine how long the exposure to infra-red may be delayed, following the end of a single light period, in order still to obtain inhibition of germination. Fourteen groups of seeds were exposed to red light for 4 hours. Each group was then subjected to infra-red radiation (3000 ergs/cm²/sec.) for 30 minutes at various times

Table 9. *Effect of chilling on germination of seeds afterwards transferred to a temperature of 14° C.*

8 hour photoperiods		Dark	
Chilled	Unchilled	Chilled	Unchilled
95 %	16 %	95 %	0 %

after the light exposure. The seeds were kept in the dark at 20° C. before and after irradiation with infra-red. Table 8 shows the results which were obtained after 10 days storage in the dark.

It is concluded from these results that when infra-red treatment is given up to 10 hours after exposure to red light, the germination processes are still partially or entirely reversible. After 12 hours, however, these processes are no longer under the influence of inhibitory radiations and germination takes place. The results are similar to those obtained by Evenari and Neumann (1953) who found that in lettuce seed, after a lapse of 10 hours the stimulatory effect of red light cannot be appreciably reversed.

Effect of chilling (»Stratification«)

The effect of low temperature treatment ('stratification') on seed germination has been known for some time and is widely used as a means of breaking the dormancy of certain seeds. The effect of chilling the seeds of *Betula pubescens* was tested in the following experiment.

Seeds were kept on moist filter papers in the dark at temperatures ranging from 1—5° C for 4 weeks. These 'stratified' seeds were then divided into two groups, one being placed in the dark at 14° C, the second being exposed to 8 hour daily photoperiods (light from 100-watt filament lamp at intensity of 45 foot candles) at a temperature of approximately 14° C. 'Controls' consisting of unchilled seeds were kept in the same conditions. The results were obtained after 14 days (Table 9). It is apparent from these results that there is no longer a light requirement for germination after chilling.

Germination of excised embryos

Experiments described above have demonstrated that germination of *Betula* seeds can be controlled by the photoperiodic conditions to which they are exposed. The question therefore arises, as to whether isolated embryos exhibit a similar type of photoperiodic response. Preliminary experiments showed, however, that isolated embryos germinated equally well in both long- and

short-days. It was, therefore, necessary to determine whether excised embryos exhibit any light requirement at all.

Embryos were dissected out of seeds which had been allowed to soak in the dark for 20 hours. Although the dissection was carried out in the light, no embryo, either when isolated or when in the seed, was exposed to light for more than 1 minute. After dissection the embryos were irradiated with infra-red (3000 ergs/cm²/sec.) for 30 minutes. Two groups of embryos, with 20 embryos in each group were thus obtained. One group of embryos was then placed in the dark at 15° C and the other in the dark at 20° C. After 13 days those embryos at the higher temperature had germinated 85 per cent, whilst those at 15° C gave 75 per cent germination. Similar results were obtained in confirmatory experiments.

Since the embryos had been exposed to 1 minute of illumination during dissection, it cannot be concluded that they would germinate in the complete absence of light. It is clear, however, that removal of the pericarp and endosperm markedly affects the response to light, for previous experiments have shown that seeds exposed to light for as long as 24 hours and then stored in the dark at 15° C give an extremely low percentage germination, and seeds exposed to light for 15 minutes and then stored at 15° C do not germinate at all (p. 307). It is therefore apparent that an abnormally high germination figure has been obtained with excised embryos.

These observations suggest that, in the intact seed, the pericarp or endosperm exert an inhibitory effect on germination. This inhibitory effect appears to be located chiefly in the pericarp, since in a further experiment, seeds from which the pericarp alone had been removed (leaving the endosperm intact) gave a high percentage germination, after one minute's illumination followed by dark storage at 15° C.

Discussion

The foregoing experiments provide clear evidence that the unchilled seeds of *Betula pubescens* show marked photoperiodic responses in relation to germination. After this work had been completed, it was reported by Isikawa (1954) that seeds of certain herbaceous species also show photoperiodic effects, greater germination being obtained in long days than in short days. These are the first reported instances of photoperiodic control of germination. Borthwick and his co-workers (1952) showed that the action spectrum for germination of light-sensitive lettuce seed is the same as that for the photoperiodic control of flowering and for other phenomena such as internode extension. They concluded that the same photoreaction is involved both in the

photoperiodic control of flowering and in light-sensitivity of seeds, but in lettuce-seed photoperiodic effects are not observable, since a single light exposure is sufficient to bring about germination.

Although facilities were not available for determining the action spectrum for the photoperiodic control of germination in birch seed, presumably the same pigment system is involved as in lettuce, since the reversibility of the effects of red-light by infra-red radiation is seen in both species.

It is of interest to compare the phenomena in birch seeds with those occurring in leafy plants of both herbaceous and woody species, and in dormant resting-buds. It is well-known that in long-day and short-day herbaceous species, both high intensity and low intensity ('secondary') photo-reactions are involved. The high intensity reaction appears to involve photosynthesis (Liverman and Bonner, 1953). There is no evidence for any reaction in birch seeds involving high light-intensities and presumably we are here dealing with only the 'secondary' light-reaction of herbaceous species, as appears to be the case in resting-buds of *Fagus sylvatica* (Wareing, 1953).

One of the characteristic features associated with the photoperiodic control of flowering in herbaceous species, is the occurrence of a well-marked 'critical' dark period, flowering in short-day plants being dependent upon exposure to cycles which include a certain minimum period of unbroken darkness, while in long-day plants flowering is inhibited if the duration of the dark periods exceeds the 'critical' value. There appears to be no well-marked critical dark period controlling germination of birch seed, however, since the percentage germination shows a *gradual* reduction as the length of the dark period is increased (p. 305). In this respect, also, the response of birch seeds resembles that of resting buds of *F. sylvatica* (Wareing, loc cit.). On the other hand, *leafy* seedlings of certain woody species do show a well-marked critical 'daylength'. Thus, seedlings of *Robinia pseudacacia* remain in active growth continuously for many months under long days, but rapidly become dormant under daily photoperiods of less than 12 hours (Moshkov, 1932; Kramer, 1936). Leafy seedlings of *B. pubescens* also show a well-marked critical daylength of about 12 hours (Wareing, unpublished). The occurrence of a well-marked critical daylength thus appears to be dependent on the presence of mature leaves. In leafy plants there appears to be a *positive* 'dark' reaction, taking place only in the absence of light, and which promotes flowering in short-day plants and inhibits flowering in long-day plants. There is no evidence for a positive dark reaction in birch seeds however. Firstly, an increased temperature during the dark period generally enhances the effect of the latter in leafy plants. Thus, in long-day plants an increased temperature during the dark period retards or even completely suppresses flowering (Lang and Melchers, 1943), as would be expected if there is an active, inhibitory

dark process having a positive temperature coefficient. If there were an active dark process (inhibitory of germination) in birch seeds, a high temperature should increase the inhibitory effect whereas the reverse is true (p. 309). It may tentatively be concluded, therefore, that the active dark process occurring in the leaves of herbaceous plants is absent in birch seeds.

On the other hand, the observation that isolated birch embryos have no light-requirement for germination (p. 309) suggests that in the intact seeds germination is inhibited by the pericarp, and that the embryo is enabled to overcome this inhibition by exposure to light. On this hypothesis, the photoperiodic responses are to be interpreted primarily in terms of the *promotive effect of light*, the effect of long dark periods being a passive one, resulting from the decay of some light-product, so that the rate of summation of this product over successive light-exposures is reduced.

The effects of temperature upon the photoperiodic responses are complex and difficult to interpret. It seems clear, however, that the reduced light-requirements observed at 20° C result from the promotive effect of the higher temperature on certain processes initiated by the photo-reaction (p. 308). On the other hand, the light requirement is lost when the seed is chilled at 1—5° C for several weeks. In this respect the seeds show responses similar to those of resting-buds of *B. pubescens*, which will only break dormancy when exposed to long photoperiods if unchilled, but will expand even in complete darkness after chilling (Wareing, 1954).

It seems unlikely that these photoperiodic responses of unchilled birch seeds are of importance under natural conditions, since they will normally be exposed to chilling conditions during the winter. Nevertheless, unchilled birch seeds present very favourable material for the study of photoperiodism under controlled conditions.

Grateful acknowledgement is made to Dr. H. J. J. Braddick of the Physics Dept., Manchester University, for advice and assistance in connection with the calibration of the photometric equipment.

Summary

1. Unchilled seeds of *Betula pubescens* are shown to be light-requiring
2. At a temperature of 15° C the unchilled seeds show marked photo-periodic responses in relation to germination, long days resulting in a higher percentage germination than short days.
3. The response depends upon the durations of both the light- and the dark-periods.
4. At a temperature of 20° C the 'photoperiodic' nature of the response is lost and a high germination percentage is obtained under both short-day

- and long-day conditions. At this temperature a single light-exposure is sufficient to give a high germination figure.
5. The effect of increased temperature appears to be primarily upon certain processes initiated by a preceding period of illumination.
 6. The promotive effect of a single exposure to red light can be reversed by infra-red radiation.
 7. In unchilled seed the pericarp appears to exert an inhibitory effect upon germination, which is overcome by light-exposure.
 8. *Chilled* seeds of *B. pubescens* show no light requirement.

References

- Black M. & Wareing P. F.: Photoperiodic control of germination in seed of birch (*Betula pubescens*, Erh.). — *Nature*. 174: 705. 1954.
- Borthwick, H. A., Hendricks S. B., Parker M. W., Toole E. H. & Toole V. K.: A reversible reaction controlling seed germination. — *Proc. Nat. Acad. Sci.* 38: 662. 1952.
- , — Toole E. H. & Toole V. K.: Action of light on lettuce seed germination. — *Bot. Gaz.* 115: 205. 1954.
- Evenari M. & Neumann G.: The germination of lettuce seed. III. The effect of light on germination. — *Bull. Res. Council Israel*. 3. No. 1—2. 1953.
- Isikawa S.: Light sensitivity against germination. I. »Photoperiodism» of seeds. — *Bot. Mag. Tokyo*. 67: 51. 1954.
- Kramer P. J.: The effect of variation in length of day on the growth and dormancy of trees. — *Plant Phys.* 11: 122. 1936.
- Lang A. & Melchers G.: Die Photoperiodische Reaktion von *Hyoscyamus niger*. — *Planta* 33: 653. 1943.
- Liverman J. L. & Bonner J.: Biochemistry of the photoperiodic response. The high-intensity light reaction. — *Bot. Gaz.* 115: 2. 1953.
- Moshkov B. S.: Photoperiodicity of trees and its practical importance. — *Bull. App. Bot. Gen. and Pl. Breeding. Ser. A. Soc. Pl. Ind.* 2: 108. 1932.
- Olmsted C. E.: Experiments on photoperiodism dormancy and leaf age and abscission in sugar maple. — *Bot. Gaz.* 112: 365. 19
- van der Veen R.: Influence of daylength on the dormancy of some species of the genus *Populus*. — *Physiol. Plant.* 4: 35. 1951.
- Wareing P. F.: Growth studies in woody species. V. Photoperiodism in dormant buds of *Fagus sylvatica* L. — *Physiol. Plant.* 6: 692. 1953.
- Growth studies in woody species. VI. The locus of photoperiodic perception in relation to dormancy. — *Physiol. Plant.* 7: 157. 1954.

Carbon Dioxide as Carbon Source and Narcotic in Photosynthesis and Growth of *Chlorella Pyrenoidosa*

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1. Introduction

As shown by Österlind 1950 and by Briggs and Whittingham 1952 free CO_2 is the only carbon source utilized by *Chlorella pyrenoidosa* for photosynthesis. Besides being the carbon source, if it is present in very high concentrations, carbon dioxide can act as a narcotic for *Chlorella* and for other plant species (Ballard 1941, Österlind 1949 and Steemann Nielsen 1953). The influence of CO_2 on photosynthesis and growth has thus several aspects.

Although the unicellular alga *Chlorella pyrenoidosa* is commonly cultivated in many laboratories all over the world, its growth rate in response to varying carbon dioxide concentration has received but limited attention. Davis, Dedrick, French, Milner, Myers, Smith and Spoehr 1953 showed that the growth rate of *Chlorella* was not significantly influenced by differences in CO_2 concentrations ranging from 0.56 to 4.43 per cent (by volume). However, no experiments were made below the relatively high concentration of 0.56 per cent. Experiments at lower CO_2 concentrations were made by Österlind 1950. The results of these experiments seem, as will be shown below, to have been influenced by the rather dense algae suspensions used.

When Otto Warburg about 1920 introduced the unicellular alga *Chlorella*, his manometric methods in experiments on photosynthesis opened a wide field. Cellular suspensions of this alga can be treated nearly like a chemical compound. This advantage has induced many biochemists to become inte-

rested in the chemical problems associated with photosynthesis thus furthering our present knowledge of this process.

Unfortunately there has been a drawback in using *Chlorella* in such a way. The concentrations of algae used in most *Chlorella* experiments have been so high that non-ecological conditions have prevailed. While for many experiments this has had no adverse influence on the results, experiments relating to the influence of carbon dioxide concentration on growth and photosynthesis have often become unrealistic. For example Warburg 1952 believes that a CO_2 concentration of 5 per cent is necessary to produce a maximum quantum yield in photosynthesis as well as a maximum rate of growth. He writes further: »Wächst sie (*Chlorella*) in der freien Natur schneller, so erhält sie Kohlensäure aus anderen Quellen als der Atmosphäre, oder das Wasser, in dem sie wächst, enthält organische Stoffe» (p. 340).

Whereas experiments concerning the rate of growth of *Chlorella* at low CO_2 concentrations have been nearly lacking, experiments on the rate of photosynthesis have been made to a much higher extend. According to Emerson and Green 1938, Whittingham 1952, Briggs and Whittingham 1952, and Steemann Nielsen 1953, the maximum rate of photosynthesis is achieved at a CO_2 concentration of about 0.01—0.03 per cent. A concentration of 0.03 per cent is found in water in equilibrium with the atmosphere at 15°C . With the exception of the 3rd. and the 4th articles the experiments were made without presence of HCO_3^- ions. The results of the experiments on photosynthesis made by Österlind 1951 and by Rosenberg 1954 do not apparently support this viewpoint. They will be discussed in sections 4 and 5. In a review of photosynthesis Lumry, Spikes and Eyring 1954 state (p. 315): »Recent studies indicate that the concentration of carbon dioxide required for maximum photosynthesis rates may be higher than was generally believed.» A reconsideration of the problem thus seems highly necessary. Warburg's claim of the necessity of using very high CO_2 concentrations in order to obtain maximum quantum yields in photosynthesis gives the problem of CO_2 dependency in photosynthesis a much wider scope. A detailed criticism of the recent articles indicating a relatively high CO_2 requirement was therefore found necessary.

It should, however, be mentioned, that neither according to Österlind nor to Rosenberg a CO_2 concentration of about 5 per cent — as claimed by Warburg — is necessary for obtaining CO_2 saturation in *Chlorella* photosynthesis and growth. According to them about 0.2—1.0 per cent is sufficient in saturated light, and of course still less in non saturated light.

In a previous article — Steemann Nielsen 1952 — it was pointed out that the thickness of the layer in the plant across which CO_2 dissolved in a fluid has to diffuse — the distance from the surface to the chloroplasts

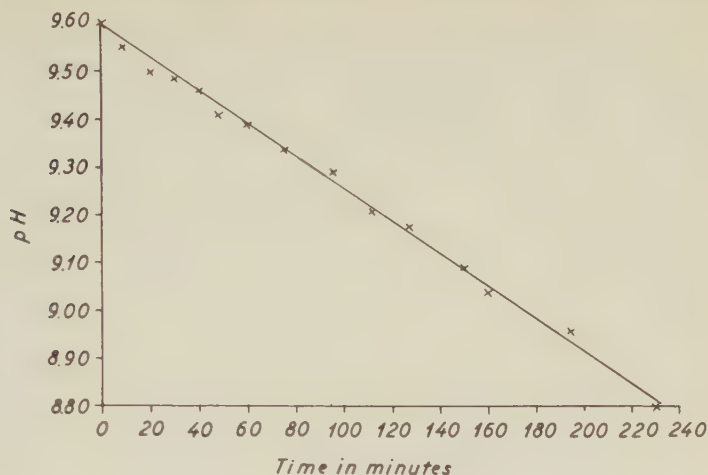


Figure 1. Change of pH with time in a carbonate-bicarbonate buffer (2 mmol/l) aerated with ordinary air.

farthest away — first of all determines the minimum CO_2 concentration necessary for producing the maximum rate of photosynthesis. Only submerged aquatic plants having thick leaves or thalli require a really high concentration of free CO_2 when this carbon source is the only one available. In a small plankton alga like *Chlorella*, where the distance for CO_2 to diffuse is very limited, a low CO_2 concentration in the surrounding water theoretically should be sufficient for establishing a maximum rate of photosynthesis.

It is reasonable — but not obvious — to imagine that photosynthesis and growth are both similarly dependent on the concentration of CO_2 . In this article it will be shown that this is actually the case.

2. Uptake of CO_2 by a Culture Medium Aerated with Ordinary Air and some Measurements on the Rate of Photosynthesis

Chlorella is commonly grown in an aerated culture medium. Drechsel gas washing bottles have been widely used for this purpose (see Burk and Warburg 1951).

Figure 1 shows how pH varies with time in 150 ml. of an aerated carbonate buffer solution (no algae present). The pH at the start was 9.60 and the concentration of total CO_2 2 mmol per litre. The temperature was 23 °C. A Drechsel gas washing bottle aerated with ordinary air at a speed of 60 ml. per minute was used. It took nearly 4 hours to decrease the pH to 8.80.

The dependency of the rate of CO_2 uptake on pH in the present experiment is shown in Figure 2. The rate of CO_2 uptake for a definite time is computed

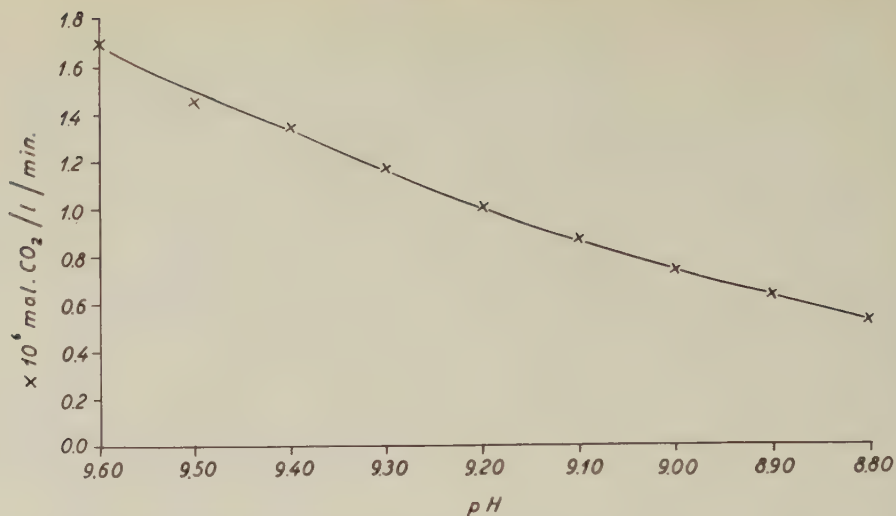


Figure 2. Dependence of CO_2 uptake on pH of a carbonate -bicarbonate buffer (2 mmol/l) aerated with ordinary air.

by taking the difference in the amount of total CO_2 before and after. It is possible to estimate total CO_2 by means of pH, temperature and the concentration of the cations added together with the bicarbonate or carbonate ions (=carbonate alkalinity, Buch 1945). Rosenberg 1954 offers a table which at 25°C can be directly used for the estimation of total CO_2 at the cation concentration 2 mmol/l.

The rate of CO_2 uptake in the present experiment was 1.7×10^{-6} mol per litre per minute at pH 9.60. As the experiment progressed, the rate of CO_2 uptake dropped constantly. At pH 8.80 it was 0.5×10^{-6} mol. At a pH about 8.5 the CO_2 uptake would have stopped completely, the solution now being in equilibrium with atmospheric air. The air blown through the Drechsel bottle contained 13.8×10^{-6} mol CO_2 per litre. As the speed was 60 ml per minute 29 per cent of the CO_2 in the air was absorbed by the buffer solution at pH 9.60, 20 per cent at pH 9.30, 12 per cent at pH 9.00, and 9 per cent at pH 8.80.

By using small bubbles produced by introducing the air through a filter of sintered glass a better CO_2 uptake is obtained. By increasing the speed of the airflow the increase in the rate of CO_2 uptake, however, is counteracted (see p. 328).

The overall reaction during the present experiment was going on slowly. The time processes $\text{CO}_2 + \text{H}_2\text{O} = \text{H}_2\text{CO}_3$ and $\text{OH}^- + \text{CO}_2 = \text{HCO}_3^-$, both of which are pH dependent, can only have been of limited importance as the

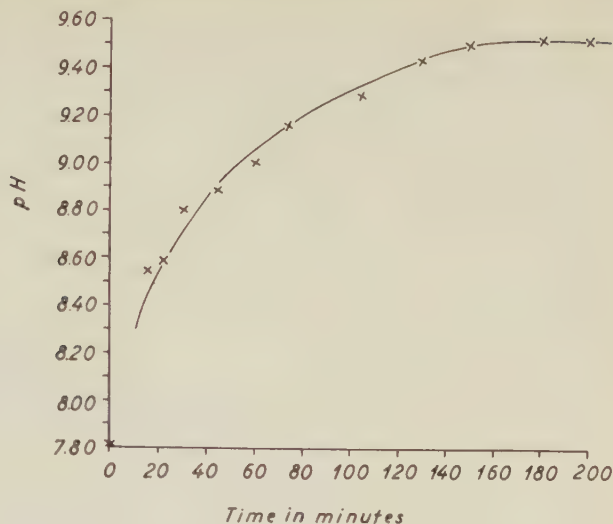


Figure 3. Change of pH with time in an illuminated suspension of *Chlorella* in a carbonate—bicarbonate buffer (2 mmol/l) aerated with ordinary air.

speed of the overall reaction was so slow. The rate of CO_2 uptake is presumably primarily regulated by the momentary difference in the concentrations of CO_2 in the solution and in the air. This difference is dependent on pH.

Figure 3 illustrates an experiment where centrifuged and washed *Chlorella pyrenoidosa* cells from a normal culture were suspended in 150 ml of a solution of KHCO_3 the concentration of which was 2 mmol per litre. Some additional ions were present in very small concentration (Ca^{++} , Na^+ , Cl^- , SO_4^{--}). The pH at the start was 7.80. The same Drechsel bottle — in a temperature bath at 23°C — was used as in the above mentioned experiment. It was illuminated by about 8,000 lux. Ordinary air at a speed of 60 ml per minute was used for aeration. The curve shows how pH varied during the first 200 minutes.

In Figure 4 the rates of assimilation of CO_2 by the algae during the experiment are shown. The crosses give the observed rates directly. The circles and the solid line give the rates if corrections are made for growth during the experiment (doubling of the population in about 12 hours).

The rates of CO_2 assimilation by the algae are computed in the following way: As pH increased during the experiment the total rate of CO_2 assimilation by the algae is the sum of the CO_2 uptake from the air flow (to be deduced from the curve in Figure 2) and the amount of withdrawn CO_2 responsible for the increase in pH. This amount is computed from $\frac{d \text{pH}}{d t}$ as shown above.

In Figure 5 the same experiment as in Figure 4 is shown. The CO_2 concentrations of the water now appear as the abscissa. The saturation plateau is

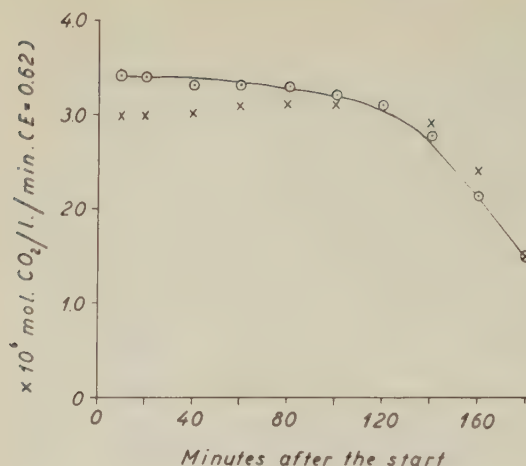


Figure 4. Rates of apparent CO_2 assimilation during the experiment shown in Fig. 3.

reached at a CO_2 concentration of about 0.01 per cent. At a concentration of 0.0015 per cent half the maximum rate of photosynthesis was found. This is in perfect agreement with the results obtained by Whittingham 1952 and by Briggs and Whittingham 1952. In these works other methods for measuring photosynthesis were used.

As shown in Figure 3 the increase in pH in the present experiment stopped at 9.54. Photosynthesis here is thus only supported by the CO_2 taken up continuously from the air blown through the culture medium. Under the present experimental conditions $1.5 \times 10^{-6} \text{ mol CO}_2 / \text{l/min.}$ is thus the maximum rate of photosynthesis of *Chlorella pyrenoidosa* at the final pH.

At the present concentration of algae the photosynthetic rate at the final pH was 44 per cent of the maximum rate. If the concentration of algae had been about 50 times as high — the normal concentration in most laboratories when cultivating *Chlorella* — the photosynthetic rate under the present conditions would have been only about 1 per cent of the maximum rate. It is thus easy to understand that a CO_2 concentration in the air flow about 100 times higher than found in ordinary air would be required to get a maximum rate of growth.

The density — E value — of the culture used in the experiment in question was 0.616. $E = \frac{1}{l} \log \frac{I_0}{I}$ where l is the depth of the culture in cm (=inner diameter of the colorimeter tube), I_0 the deflection of the galvanometer when the tube is filled with distilled water, and I the deflection when the tube is filled with the culture. An EEL colorimeter with a blue filter was used. The method of measuring E is in principle the same as used by Österlind 1949 and 1950.

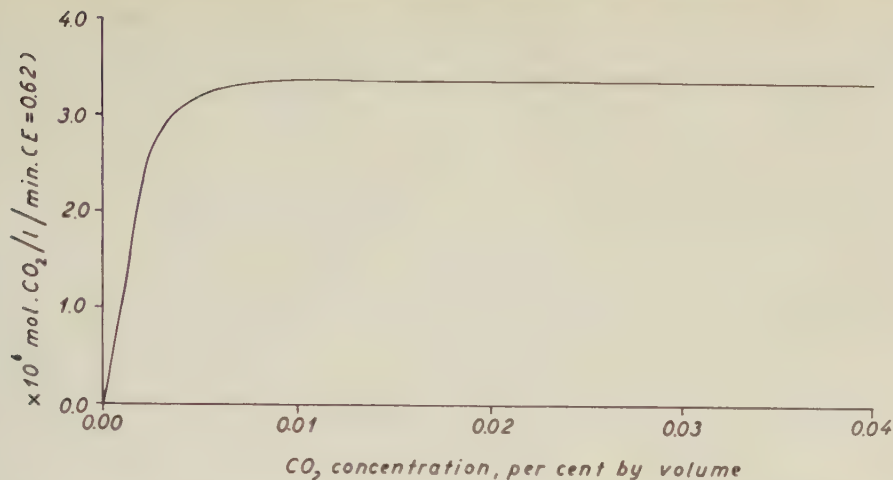


Figure 5. Dependence of rate of photosynthesis on the CO_2 concentration. According to the experiment shown in Fig. 3.

In Figure 6 an experimental series is given showing the dependence of rate of photosynthesis on the CO_2 concentration. The carbon-14 method for measuring rate of photosynthesis was used (Steemann Nielsen 1952).

Chlorella pyrenoidosa cells grown in the ordinary way were after centrifugation suspended in 50 ml of a solution of 1.5×10^{-3} mol KHCO_3 + small quantities of neutral salts. To this was added 1 ml of a solution of $\text{NaH}^{14}\text{CO}_3$ — 2×10^{-3} mol/l giving per ml 1.5×10^6 counts per minute as measured by an end-window Geiger tube with a window radius of 7.5 mm. The initial pH was 8.3. The light intensity was about 10,000 lux. As the density of the algae was low, the illumination of the single cells was even. The temperature was 24°C . A temperature bath was used.

At time intervals 5 ml samples of the algae suspension were withdrawn and the algae were filtered off. A millipore filter aerosol was used. After measuring the counts per minute the rate of photosynthesis was estimated. During the experiment both the concentration of total CO_2 and free CO_2 constantly decreased. The concentration of total CO_2 at any time was determined by subtracting the already photosynthesized CO_2 from the original amount of total CO_2 . By means of total CO_2 , carbonate alkalinity ($= 1.5 \times 10^{-3}$ mol/l), and the apparent dissociation constants of carbon dioxide in water at the present temperature, pH can be estimated which again allows a determination of the concentration of free CO_2 .

For convenience sake the equilibrium values for $\frac{d(\text{C})}{d \text{ pH}}$ given in Table 1

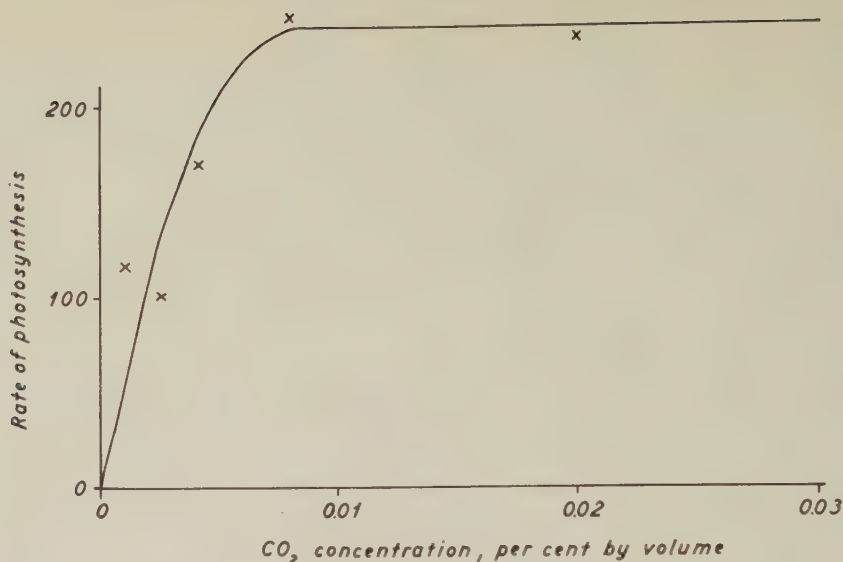


Figure 6. Dependence of rate of photosynthesis on the CO_2 concentration. *Chlorella*, Carbon-14 method.

by Rosenberg 1954 were used for computing the changes in pH. The concentrations of free CO_2 used in each single determination is the intermediate between the concentration at the start and at the end.

The curve in Figure 6 shows the results of the experiment. From a concentration of about 0.01 per cent free CO_2 the rate of photosynthesis is independent of CO_2 concentration. The curve is nearly identical with the curve obtained with the other method of measuring rate of photosynthesis shown in Figure 5.

Using 5 different methods it has thus been shown that *Chlorella pyrenoidosa* normally only requires a very low CO_2 concentration for photosynthesizing at maximum speed. These 5 methods are: 1) the manometric method used by Briggs and Wittingham 1952, 2) the infra-red gas analyser method used by Wittingham 1952, 3) the oxygen method used by Steemann Nielsen 1953 4) the glass electrode method recording CO_2 uptake used in the present work and 5) the carbon-14 method used in the present work too. As to be shown in the next section experiments on growth in *Chlorella* show just the same CO_2 dependence as photosynthesis.

It should now be possible to state with a very high rate of certainty that normally grown *Chlorella pyrenoidosa* cells only require a very low CO_2 concentration for producing maximum rate of photosynthesis. The concen-

tration is at most as high as found when water is in equilibrium with atmospheric air. Not absolutely healthy *Chlorella* cells may possibly sometimes require a slightly higher CO_2 concentration.

3. Growth Experiments with *Chlorella* at Different CO_2 Concentrations

If very weak densities of *Chlorella* are used growth experiments can be made without aeration. Under these circumstances it is of course impossible to use photometric methods for measuring growth. Instead the algae have to be counted microscopically. Counting of a pure culture while tedious, is none the less as precise as the photometric method.

The following counting method was used: 10 ml. of the culture in tubes with a tapered bottom were centrifuged for 5 minutes at 3,000 revolutions per minute. The supernatant was poured to waste leaving a residue of 0.20 ml. A bloodcounting chamber was used to determine the number of cells in the remaining 0.20 ml.

Figure 7 illustrates a series of growth rate measurements induced by varying the concentration of carbon dioxide. The basic nutrient solution used corresponded to Solution C, Österlind 1949 page 41. The carbonates were of course omitted. Varying amounts of NaHCO_3 were instead added. The pH varied from 7.9 to 8.1 and the concentration of free CO_2 varied from 0.015 to 0.15 per cent. The initial concentration of *Chlorella pyrenoidosa* in all bottles (15 ml. capacity with glass stoppers) was adjusted to 14 cells per mm^3 . During 23 hours under 9,000 lux at 20°C the amount of algae at all concentrations of CO_2 increased to about 100 per mm^3 . In Fig. 3 the growth rate is shown expressed as the constant n . According to Österling 1949

$$n = \frac{2.303}{t_1 - t_0} \times \log \frac{A_{\text{beg.}} + A_1}{A_{\text{beg.}}}$$

where $t_1 - t_0$ = duration of the experiment, expressed in hours, $A_{\text{beg.}}$ = concentration of algae at the start, A_1 = concentration of the algae at the end of the experiment.

According to Figure 7, the concentration of carbon dioxide in water in equilibrium with ordinary air (tension about 0.03 per cent) is sufficient to produce maximum growth rates at pH 8. This is in accordance with the experiments mentioned above concerning the rate of photosynthesis in relation to the concentration of free carbon dioxide.

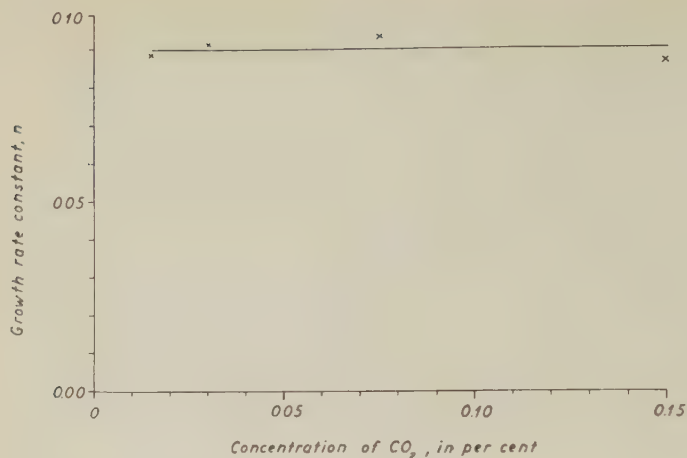


Figure 7. *Dependence of rate of growth in Chlorella on the CO₂ concentration.*

4. Remarks on Experiments made by Österlind

The results of the experiments described above do not agree with those published by Österlind 1950. According to his experiments growth at a CO₂ concentration of 0.03 per cent (pH 8) is only $1/10$ — $1/5$ of the maximum rate observed.

When growing another unicellular alga, *Scenedesmus*, Österlind 1949 paid adequate attention to the density of the culture. An entire section, pages 46—50, is presented to prove at which densities of the algae the supply of CO₂ from the air is sufficient to maintain a constant concentration of the carbonic acid components. Under the growth conditions used it was stated that the *Scenedesmus* cells do not suffer from CO₂ deficiency when the culture is aerated with ordinary air at a rate of 30—60 ml. per minute provided that an E value of 0.04 is not exceeded.

When presenting this *Chlorella* experiments Österlind 1950 unfortunately neglected to mention the density of his cultures, but relying on his experiences with *Scenedesmus* it would seem unlikely that he should have used a density higher than $E=0.04$. However, one important alteration in the method of growing algae was introduced. In the previous *Scenedesmus* experiments, tubes with an inner diameter of about 18 mm and containing 10 ml of the culture were used. The depth of the culture was about 5 cm. In the *Chlorella* experiments difficulties were encountered by using tubes. Due to the production of surface active substances, the cultures foamed while being aerated. Ultimately the experiments had to be performed in 100 ml conical flasks, each containing 50 ml of the culture. The depth of the culture was about 2 cm. When using these conical flasks, the path of air bubbles rising in the culture

solution was less than half the path when the tubes were used. Furthermore the volume of the solution is five times greater in the conical flasks. The CO_2 uptake per equal volume was thus most likely about 10 per cent of the uptake in the tubes assuming both were aerated with the same volume of air.

Thus assuming Österlind used the same densities of *Chlorella* as he used in his *Scenedesmus* experiments the high CO_2 concentration required to produce maximum rates of growth is easily understood.

In a subsequent paper Österlind 1951 published some manometric experiments on the dependency of the rate of photosynthesis upon the concentration of free carbon dioxide. According to these experiments the rate of photosynthesis is directly proportional to the concentration of CO_2 up to at least 0.082 mmol per litre (=0.16 per cent), thus indicating that rather high concentrations of CO_2 is necessary to obtain maximum rates of photosynthesis.

According to the experiment illustrated in Fig. 4 of this article, 3.4×10^{-6} mol CO_2 were assimilated per litre per minute at an algal density of $E=0.616$. As E was 0.13 in Österlind's experiment the rate of photosynthesis (the light intensity was apparently the same) was about 0.8×10^{-6} mol CO_2 per litre per minute. A concentration of CO_2 of e.g. 21.3 mmol per litre (=0.05 % CO_2) could thus maintain a maximum rate of photosynthesis for only 27 minutes.

As the duration of the experiments was 50 minutes, it would appear that the diffusion from the liquid to the gas state is excessively slow if Österlind's modification of the manometric method is used. It seems not to be possible to employ this modification for the aim in question.

5. Remarks on Experiments made by Rosenberg

Rosenberg 1954 described a continuously recording glass electrode apparatus for measuring photosynthetic rates and changes in the concentration of dissolved carbon dioxide. It was used for the measurement of transient rates of photosynthesis by suspensions of *Chlorella pyrenoidosa*. Rosenberg's measurements apparently showed a decline of the photosynthetic rate in high light at CO_2 partial pressures less than 1 per cent. This observation was apparently confirmed by steady state experiments in which flowing gas streams were analyzed.

The method of using the glass electrode for measurements of the rate of photosynthesis is in principle the same as employed in the experiments given in section 2 of this article. There are, however, rather serious objections to this method as used by Rosenberg. The same applies to his steady state experiments in which the flowing gas stream after bubbling through the algal medium is analyzed by the glass electrode in a special vessel containing a buffer solution.

The primary objection concerns the use of extremely dense algae suspensions — 0.01 ml algae per ml of the culture medium. About 1 per cent of the total volume thus consists of algae! Both the CO_2 conditions and the light conditions are strongly influenced by such an unnatural cell concentration.

Rosenberg's Figure 6 illustrates one of his steady state experiments. At the lowest CO_2 concentration used (0.15 %) the rate of photosynthesis was only about 40 per cent of the maximum one obtained at 1 % CO_2 . Information is unfortunately lacking about the details of the experiment. But let us consider that the maximum rate of the airflow reported (10 ml/minute) was used and that the maximum rate of photosynthesis was 3×10^{-6} moles/litre/second. This was the maximum rate stated if the glass electrode was placed directly in an algal suspension having the same density. If all CO_2 in the airflow at a concentration of 0.15 per cent was absorbed by the experimental solution (4 ml) and used in the photosynthesis of the algae, a photosynthetic rate of only 83 per cent of the maximum would be possible.

It is furthermore very unlikely that all CO_2 in the airflow was absorbed in the reaction vessel. Although the airbubbles produced were small due to the glassfilter through which the air entered the reaction vessel, the time a single airbubble stayed in the medium was very short due to the very shallow solution layer — about 5 mm deep.

An experiment was conducted in our laboratory. It clearly showed the inefficiency of CO_2 absorbtion from small airbubbles. 20 ml of a carbonate—bicarbonate buffer (0.002 m pH 9.60 was poured into a Buchner funnel with sintered glass at the bottom (Schott G 4). A flow of normal air (0.03 % CO_2) was pressed up through the pores of the sintered glass at a speed of 240 ml per minute. A glass electrode and a reference electrode were placed in the center of the funnel and pH was registered continuously. From $\frac{d \text{ pH}}{d t}$ the rate of the CO_2 uptake was determined. At pH 9.50, where the CO_2 tension of the solution was about $1/10$ of the tension in the air, 20 per cent of the CO_2 in the airflow was absorbed, at pH 9.20 14 per cent and at pH 9.00 10 per cent.

In Rosenberg's experiments pH was lower. As shown above the uptake of CO_2 decreases when pH decreases. The rate of airflow, however, was slower — 10 ml air per 4 ml solution compared with 240 ml per 20 ml = 48 ml per 4 ml. This explains why at a CO_2 concentration of 0.15 per cent in the airflow he was able to obtain a photosynthetic rate as high as 40 per cent of the maximum. The experiment on the other hand does not tell anything about the dependency of the rate of photosynthesis on the CO_2 concentration. Rosenberg mentions on p. 773: »Preliminary experiments indicated that at high light the inhibition (due to CO_2 limitation) was removed by higher concen-

trations of bicarbonate.» This indicates that the actual CO_2 pressures in the medium were much lower than stated.

Rosenberg's other method of measuring the rate of photosynthesis did not include any uptake of CO_2 from an airflow. It is never the less very near to the method used in my experiment given in Section 2. The algae concentrations used in the two sets of experiments were, however, very different. The optimum rate of photosynthesis per litre in Rosenberg's experiment at the highest light intensity used was 55 times higher than at saturating light in our experiment indicating that the algal density was about 50 times higher. The density of the algae expressed as E value (blue light filter, see p. 322) was 0.616, in our experiment. This is *eo ipso* a high density, much higher than I would recommend for normal use. It means that the intensity of the blue light at a depth of 1 cm from the surface facing the light is about 25 % of the intensity at the surface. Integrated for the whole active part of the spectrum about half of the light energy is absorbed in the first cm.

In Rosenberg's experiment the light intensity must have varied enormously from one depth to another of the algae suspension. This is clearly shown by the response to variations in the intensity of the incident light. According to his Fig. 6 the rate of photosynthesis is tripled by increasing the light intensity from 900 to 4200 foot-candles. Normally 900 foot-candles are sufficient for producing a maximum rate of photosynthesis in *Chlorella*. It can be assumed without difficulty that the light intensity at the distal extremity of the reaction vessel at the highest intensity of the incident light was below the compensation point.

As no permanent stirring was produced in the reaction cell it is difficult to say what the measurements by the glass electrode really mean. The top of the electrode was about 2 mm from the front of the reaction vessel. The algae here were thus relatively well illuminated. The most distant part of the electrode was 5 mm away. The algae here were very badly illuminated. The lack of water cooling of the reaction cell must also be mentioned. As white light from a projector bulb was used, the temperature inside the reaction cell may have been rather different from room temperature.

Furthermore the influence of the time process $\text{CO}_2 + \text{H}_2\text{O} \rightleftharpoons \text{H}_2\text{CO}_3$ cannot be fully disregarded in an experiment where all the pH changes used for computing the rate of photosynthesis (between 7.0 and 7.6) is limited to 2 minutes. Finally the rather long induction times necessary in photosynthesis when going from a high to a low CO_2 concentration — see Briggs and Whittingham 1952 and Whittingham 1952 — must seriously influence the results of experiments where the CO_2 concentration is changing rapidly.

Collectively these objections do not substantiate Rosenberg's results which differ so much from the results obtained by other investigators.

6. The Influence of very high CO_2 Concentrations on the Rate of Photosynthesis

In a previous paper — Steemann Nielsen 1953 — it was shown that very high concentrations of free CO_2 in the water may influence the rate of photosynthesis in *Chlorella*. A distinct decrease was found in saturated light at concentrations above 1 per cent. The decrease was about 20—40 per cent at a CO_2 concentration of 5 per cent. The rate of photosynthesis was on the other hand found to be constant when varying the concentration from about 0.03 to 1 per cent. It was assumed that CO_2 in a concentration of more than 1 per cent acts as a poison (or better as a narcotic — see Rabinowitch 1946 p. 300) for at least one of the enzymatic processes involved in photosynthesis. The same effect of CO_2 has been found by other workers in a variety of species — e.g. in *Scenedesmus quadricauda* by Österlind 1949.

Quite contrarily to the results in saturated light a series of experiments at low light intensities (near the compensation point) showed a pronounced increase in the rate of apparent CO_2 assimilation when the CO_2 concentration was increased above 1 per cent. Variations from 0.05 to 1 per cent did not alter the rate of photosynthesis. It was therefore suggested that in light a high CO_2 concentration may give rise to a blocking of the respiratory mechanism. This would account for the increased rate of apparent CO_2 assimilation.

In the following mainly the influence of high CO_2 concentrations on the rate of photosynthesis in saturated light will be considered. Scarcely more than some few of the enzymatic processes involved in photosynthesis are affected by CO_2 in excess. The entire photosynthetic process in saturated light is apparently not always limited by the same enzymatic process. Differences from one experiment to another may therefore be expected regarding the CO_2 concentration above which the rate of photosynthesis starts to decrease. In one of our series of experiments the decrease in the photosynthetic rate only was found at concentrations above 5 per cent.

The results of the experiments correspond very well with those obtained by Ballard 1941 using leaves of terrestrial plants. This worker furthermore made the interesting observation that the narcotic effect of CO_2 started at a much lower concentration at a low temperature — 6°C — than at a higher temperature.

By making a rather long sequence of *Chlorella* experiments concerning the influence of varying CO_2 concentrations in saturated light 3 series turned out to give a somewhat special picture at a low pH whereas a normal behavior was found at a higher pH. At the same time the maximum rate of photosynthesis in saturated light was — in contrast to normal conditions — found to be pH dependent. It must be pointed out that these 3 series all were made

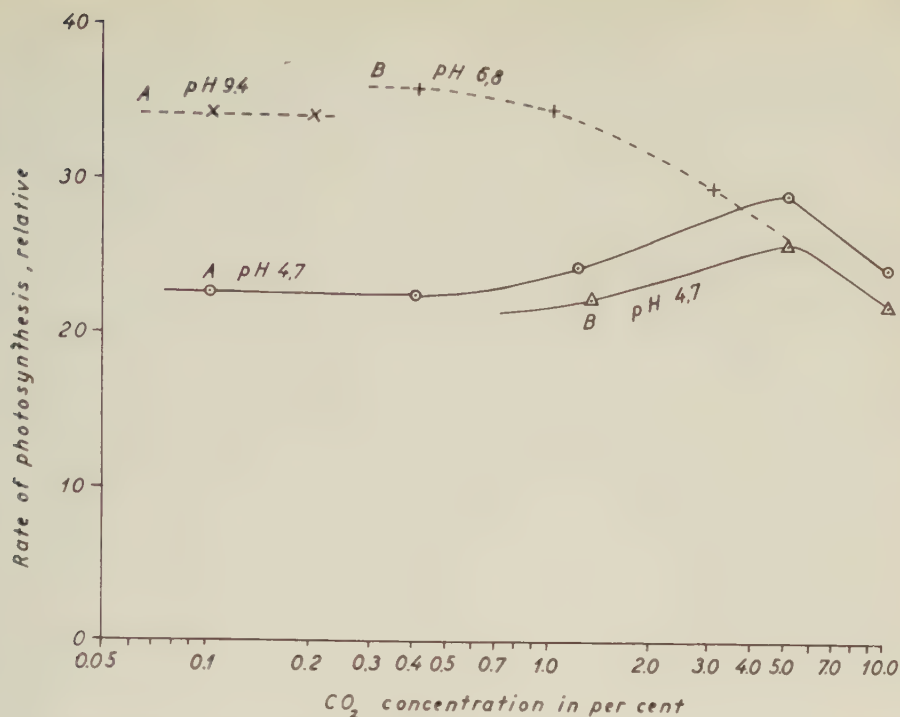


Figure 8. Rates of photosynthesis in *Chlorella* in saturated light at different pH as a function of the CO₂ concentration.

in the same period — during a single week. The culture medium for growing the algae previous to the experiments had been prepared at the same time. If some special factor had been introduced, it must have been by accident.

The methods for cultivation and for measuring the rate of photosynthesis — oxygen determinations by Winkler — were the same as described in Steemann Nielsen 1953. All measurements were made in duplicate. The density of the algae was always kept so low that all algae during an experiment were exposed to practically the same light intensity. The same was true concerning the experiments described in the 1953 article where by a mistake nothing was stated about the density of the algae.

In the two series shown in Figure 8, experiments were made both at a high and at a low pH, in series A at pH 9.4 and pH 4.7, in series B at pH 6.8 and pH 4.7. The light intensity was 9,000 Lux, temperature 21° C. The rates of photosynthesis are given relatively, but they are all computed for the same E value — see p. 322, for absolute rates see p. 332. In series A at pH 9.4 the saturation plateau for CO₂ was reached at a concentration below

0.1 per cent. Real high concentrations of free CO_2 can not be employed at this pH because of the abnormally high concentrations of HCO_3^- and CO_3^{--} . In series B at pH 6.8 it was possible to use concentrations of free CO_2 up to 3 per cent. The usual decrease in the photosynthetic rate at concentrations above 1 per cent was found.

At pH 4.7 series A covers CO_2 concentrations from 0.1 to 10 per cent. In series B at pH 4.7 experiments were done only in the range 1.3—10 per cent. But here the results were exactly as in series A. The rate of photosynthesis did not change by varying the CO_2 concentration from 0.1 to 0.4 per cent. The CO_2 saturation plateau was thus reached at a concentration below 0.1 per cent. In both series, however, the rate of photosynthesis at the saturation plateau at pH 4.7 was far below the rate obtained at the higher pH. At pH 4.7 in series A the rate at the plateau was 67 per cent of the rate at pH 9.4, in series B the rate at pH 4.7 was only 57 per cent of the rate at pH 6.8.

This dependency on pH is as already mentioned not usually found in *Chlorella pyrenoidosa*. Emerson and Green 1938 thus found no influence on the rate of photosynthesis in the pH range 4.6—8.9 and according to Fig. 1, Steemann Nielsen 1953 the rates were identical at pH 4.2 and 9.4. The pH dependency observed in series A and B was also found in the third series made at the same time. It must be stressed that the photosynthetic rates at a high pH in these 3 series measured per density of algae were as high as found in series showing no pH influence. Computed for $E=0.62$, the density of algae found in the experiment described in Section 2, the maximum rate at pH 6.8 in series B is 2.8×10^{-3} mol CO_2 /l./min., which is nearly the same as found in the above mentioned experiment.

As mentioned above the algae in series A and B showed another peculiar behavior. At CO_2 concentrations about 1—5 per cent a definite increase in the photosynthetic rate at pH 4.7 was found (see Figure 8). It was just the opposite of what was found at the higher pH in the same series. In these series there is presumably a connection between the pH dependency and the stimulating effect of high CO_2 concentrations at a low pH.

In general terms the explanation may be this. For some reason a process which has a decreasing effect on the rate of photosynthesis is stimulated at a low pH in these series. At a high CO_2 concentration, this process, however, is blocked. The rate of photosynthesis thus increases. The process in question may simply be the invasion of H^+ ions into the protoplasm, which very likely could be influenced by CO_2 in excess through blocking of the centers in the plasma membranes responsible for uptake of ions into the cell. It may instead be some sort of photooxidation, induced by the low pH, attacking enzymes involved in photosynthesis. A decreasing effect of CO_2 in high concentrations on photooxidation seems to be a possibility — cmp. Franck and French 1941.

It seems on the other hand unlikely that the increase of the apparent CO_2 assimilation in the experiments in question is effected by the blocking of some process in normal respiration. The increase in the rate of CO_2 assimilation by increasing the CO_2 concentration from 1 to 5 per cent is nearly 4 times greater than the rate of respiration in the dark at the pH in question.

The combination of the two curves from series B (Figure 8) is rather impressive. At both pH it seems evident that above a CO_2 concentration of 5 per cent the same partial process blocked by the excess CO_2 is limiting the overall photosynthetic process. At pH 6.8 the same process is limiting below 5 per cent CO_2 . This is, however, definitely not the case at pH 4.7.

If CO_2 in high concentrations acts as a narcotic in the same way as the different urethans — *cmp.* Wassink, Vermeulen, Reman and Katz 1938 — it may interfere not only with the enzymatic mechanisms of photosynthesis by blocking enzymes responsible for light saturation but also with the sensitization process itself. The eventual blocking of photooxidation as mentioned above would in fact be the same as an inhibition of the transfer of excitation energy from chlorophyll.

With leaves of *Ligustrum* at 2,000 Lux, 6° C. Ballard 1941 observed a definite decrease in the photosynthetic rate when the CO_2 concentration exceeded 1 per cent. As the overall process at this low light intensity most likely must be limited by the photochemical part process, it is rather probable that CO_2 in high concentrations under certain conditions inhibits the normal transfer of excitation energy as do urethans. The initial slope of the light assimilation curve is thus altered. Wessel 1954 offers a rather probable explanation of the mechanism of such an inhibition.

The influence of CO_2 in excess may thus under certain circumstances be rather complicated at very low light intensities, the rate of apparent CO_2 assimilation being at the same time affected by an increasing and a decreasing influence. As shown above occasionally at high light intensities the interaction of two processes both affected by high CO_2 concentrations may also make the interpretation of a single experiment rather complicated.

High CO_2 concentrations as well as high algae densities should be avoided in all experiments not specially designed for investigating the effect of either high CO_2 concentrations or high algae densities. This is to be put in mind when measuring the quantum yield of photosynthesis.

Summary

Much confusion about the concentration of CO_2 necessary for maximum rate of photosynthesis and growth in *Chlorella* exists at present. It is shown

that too dense a concentration of algae used in many experiments is the main cause of the discrepancies.

Using very dilute concentrations of *Chlorella pyrenoidosa* it is shown that both photosynthesis and growth are independent of the concentration of CO_2 down to 0.01—0.03 per cent.

Three different and independent methods of measuring photosynthesis have been used by the present author to show the low CO_2 requirement in *Chlorella* photosynthesis. In addition still two other methods used by other workers have given exactly the same result.

The uptake of CO_2 by a culture medium when aerated with ordinary air was investigated and found very inefficient. By using small bubbles produced by introducing the air through a filter of sintered glass a better uptake is obtained. By increasing the speed of the airflow the increase in the rate of CO_2 uptake, however, is counteracted.

It is shown that the narcotic effect of very high CO_2 concentrations — above 1 per cent — may influence in different ways the rates of metabolism in *Chlorella*. In saturated light an enzymatic process which usually limits the overall photosynthetic process is blocked by high CO_2 concentrations whereby the rate of photosynthesis decreases.

The rate of photosynthesis in saturated light is usually independent of pH. Under certain circumstances, however, a process which affects a decrease in the rate of photosynthesis is stimulated by a low pH. This special process is inhibited by high concentrations of CO_2 . The rate of photosynthesis at low pH thus increases at high CO_2 concentrations.

Near the compensation point the rate of apparent CO_2 assimilation may increase at high CO_2 concentrations most likely due to a blocking of some part of the respiratory system.

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References

- Ballard, L. A. T.: The Depressant Effect of Carbon Dioxide upon Photosynthesis. — *New Phytologist* 40: 276. 1941.
- Briggs, G. E. & Whittingham, C. P.: Factors affecting the Rate of Photosynthesis of *Chlorella* at low Concentrations of Carbon Dioxide and in high Illumination. — *New Phytologist* 51: 236. 1952.
- Buch, K.: Kolsyrejämvikten i Baltiska Havet. — *Fennia* 68, No. 5. 1945.
- Burk, D. & Warburg O.: Ein Quanten — Reaktion und Kreisprozess der Energie bei der Photosynthese. — *Z. Naturforsch.* 6b: 12. 1951.
- Davis E. A., Dedrick J., French, C. S., Milner, H. W., Myers J., Smith, J. H. C. & Spoehr, H. A.: Laboratory experiments on *Chlorella* culture at the Carnegie Institution of

- Washington, Department of Plant Biology. Algal culture from laboratory to pilot plant, edited by John S. Burlew. — Carnegie Institution of Washington Publication 600: 105. 1953.
- Emerson, R. & Green, L.: Effect of Hydrogen-ion Concentration on *Chlorella* Photosynthesis. — *Plant Physiology* 13: 157. 1938.
- Franck, J.: Participation of Respiratory Intermediates in the Process of Photosynthesis as an Explanation of abnormally high Quantum Yields. — *Arch. Biochem.* 45: 190. 1953.
- & French, C. S.: Photo-oxidation Processes in Plants. — *Journ. Gen. Physiol.* 25: 309. 1941.
- Lumry, K., Spikes J. D. & Eyring, H.: Photosynthesis. — *Annual Rev. of Plant Phys.* 5: 271. 1954.
- Rabinowitch, E.: Photosynthesis and Related Processes. 2 Part 1. New York 1951.
- Rosenberg, J. L.: Use of a Glass Electrode for measuring Rapid Changes in Photosynthetic Rates. — *Journ. Gen. Physiol.* 37: 753. 1954.
- Steemann Nielsen, E.: Experimental Carbon Dioxide Curves in Photosynthesis. — *Physiol. Plant.* 5: 145. 1952.
- The use of Radio-active Carbon (C^{14}) for measuring Organic Production in the Sea. — *Journ. du Conseil Intern. l'Explor de la Mer.* 18: 117. 1952.
- Carbon Dioxide concentration, Respiration during Photosynthesis, and Maximum Quantum Yield of Photosynthesis. — *Physiol. Plant.* 6: 316. 1953.
- Warburg, O., Krippahl, G., Buchholz, W. & Schröder, W.: Weiterentwicklung der Methoden zur Messung der Photosynthese. — *Zeitschr. Naturforsch.* 8 (b): 675. 1953.
- Energetik der Photosynthese — *Die Naturwissenschaften* 39: 337. 1952.
- Wassink, E. C., Vermeulen, D., Reman, G. H. & Katz, E.: On the Relation between Fluorescence and Assimilation in Photosynthesizing Cells. — *Enzymologia* 5: 100. 1938.
- Wessel, J. S. C.: A Possible Function of Vitamin K in Photosynthesis. — *Rec. des Travaux Chim. des Pays — Bas.* 73. Nr. 7. 1954.
- Whittingham, C. P.: Rate of Photosynthesis and Concentration of Carbon dioxide in *Chlorella*. — *Nature* 170: 1017. 1952.
- Österlind, S.: Growth Conditions of the Alga *Scenedesmus quadricauda* with special Reference to the inorganic Carbon Sources. — *Symbolae Botan. Upsaliens.* 10 (3): 1. 1949.
- Inorganic Carbon Sources of Green Algae. I. Growth Experiments with *Scenedesmus quadricauda* and *Chlorella pyrenoidosa*. — *Physiol. Plant.* 3: 353. 1950.
- Inorganic Carbon Sources of Green Algae. III. Measurement of Photosynthesis in *Scenedesmus quadricauda* and *Chlorella pyrenoidosa*. — *Physiol. Plant.* 4: 242. 1951.

The Effect of an Antiauxin on the Indoleacetic Acid Content in Avena Coleoptiles

By

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In the Avena straight growth test used by Ingestad (3) native auxins were present due to the fact that the coleoptile sections were not washed in water before they were put in the test solutions. For this reason there was evidence that supplied growth substances interact competitively not only with each other (4, 5) but also with the native substances. In the present paper further investigations on the interaction of native and supplied substances are reported.

The effect of p-chlorophenoxyisobutyric acid (PCIB) on the growth of Avena coleoptiles and their content of extractable 3-indoleacetic acid (IAA) are studied. PCIB is a well-known antiauxin found to counteract IAA in roots by Burström (1) and in coleoptiles by McRae and Bonner (5).

Methods

Germination. Avena seedlings with glumes removed were grown in Vermiculite under constant conditions (25° C and 80 per cent humidity). They were irradiated with red light during the first 12 hours after emergence over the Vermiculite level. After about 70 hours the coleoptiles were used for the different treatments. About 30 per cent of the sowed seeds gave straight coleoptiles of suitable size.

Antiauxin treatment. 2.5—3.0 cm long coleoptiles were selected and cut to a length of 2.0 cm with the tips intact. These pieces were placed vertically with the cut ends

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Figure 1. Device for treating coleoptiles with PCIB.

— The excised coleoptiles are placed in a shallow dish containing the solution and supported by a plate of Perspex. The coleoptiles were subsequently extracted for IAA which was determined in a cylinder test (cf. the text).



down in Petri dishes and supported by a perforated plate of Perspex as shown in figure 1. The dishes had a diameter of 70 mm and contained 20 ml of a solution containing 1.6 per cent dextrose, 10^{-3} M potassium citrate buffer with pH 4.5 and in some cases PCIB as the potassium salt. The coleoptiles were treated for 18 hours in darkness and under constant conditions (25° C and 80 per cent humidity). In some experiments the lengths of the coleoptiles were then measured with the accuracy of 0.5 mm, in others the content of extractable IAA in the coleoptiles was estimated by means of paper chromatography and the Avena cylinder test. In the first case 20 coleoptiles were used in each Petri dish and in the second case a number of 50.

Chromatography. In these experiments it was desired to avoid destruction and loss of the native IAA by too many operations but, nevertheless, obtain the substance in a sufficiently pure state. After the coleoptiles were taken out of the solutions their wet cut ends were slightly pressed against a filter paper and the coleoptiles placed in wet ether, purified from peroxides. Fifteen ml ether were used per 50 coleoptiles. The material was crushed and extracted in darkness at 20° C for one hour. During the next half-hour the tissue was washed four times with ether, 10 ml each time. The ether was evaporated and the residue immediately extracted for half an hour with five applications of 5 ml chloroform at 20° C. These 25 ml of chloroform were then evaporated and the residue quantitatively transferred, by means of ether, to a strip of chromatogram paper (Munktell No. 00). The application was made in one spot, the strip was 2.5 cm wide and the chromatogram run with ascending solvent in a glass jar at 22° C and in darkness or red light. The solvent was composed of isopropanol, ammonia and water in the proportions 10 : 1 : 1. When the solvent front had ascended a distance of 18–20 cm the paper was removed and dried one hour in vacuum at 20° C. The part of the paper between the R_f values 0.25 and 0.50, corresponding to the position of pure IAA, was cut in small pieces and extracted one hour with five applications of 5 ml ether. The 25 ml extract thus obtained was successively transferred to a small beaker (diameter 2.5 cm) and the ether evaporated. To the beaker were then added 2 ml of a nutrient solution and the amount of extracted IAA measured as described below in the Avena test chapter.

In order to control that the extracted IAA was to be found between the R_f values mentioned the position of the pure compound was always tested on a separate chromatogram. The IAA spot was detected by spraying with p-dimethylaminobenzaldehyde (1 g in 75 ml N HCl).

A purification of IAA and its separation from PCIB by chromatography procedure is realizable because of the rather different R_f values of the two substances. In preliminary experiments the R_f values of the pure compounds were determined.

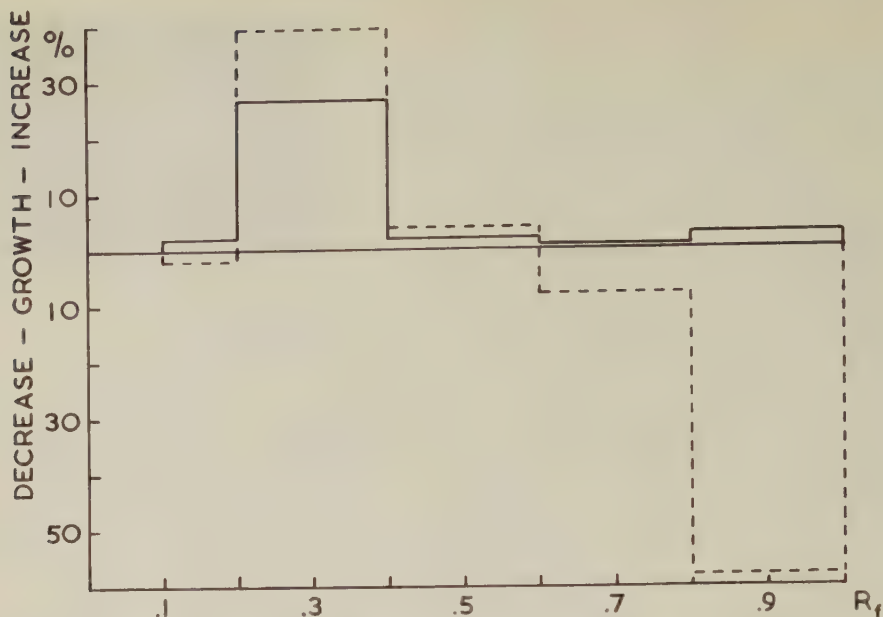


Figure 2. The effect of extracts of differently treated coleoptiles on *Avena* section growth. — Untreated (—) and PCIB-treated (---) coleoptiles were extracted and the extract chromatographed. The paper strip was cut in five sections. The separate extracts of these sections were then tested in *Avena* test. The growth is expressed as increase or decrease in respect to the growth of the control.

Five μ l of one per cent solutions were used. Whether the compounds were examined alone or mixed the R_f value of each substance was found to be constant. The PCIB spot was detected by spraying with a slightly alkaline solution of bromthymolblue and IAA with the reagent just mentioned.

The R_f value of pure IAA was 0.37 (coloured spot at R_f 0.25—0.50) and of pure PCIB 0.75 (total spot 0.70—0.80), obtained at 22° C and with the solvent isopropanol, ammonia and water (10 : 1 : 1). A good separation between IAA and PCIB by chromatography is also obtained when extracts of coleoptiles not treated and treated with PCIB are used (figure 2).

Avena test. Coleoptiles 2.5—3.0 cm in length were selected. From each a 5 mm long section was cut 3 mm from the tip. The sections were thread on thin glass needles and washed for one hour in distilled water. The washing in water was carried out to obtain low control growth. Ten sections were then placed in a small beaker (2.5 cm in diameter) containing 2 ml of a test solution of 1.6 per cent dextrose and 10^{-3} M potassium citrate buffer (pH 4.5) and added extracted IAA. After 18 hours at 25° C in darkness the section length was measured under the microscope with an accuracy of 0.1 mm.

It is shown in the next chapter that the accuracy of the test is very great in spite of the small differences. Every measurement made is recorded in table 2 and

the results are statistically analyzed. It is immediately seen that the homogeneity of the material is great with the exception of the fourth experiment where some external factor must have been altered during the test time. The authors think it is of interest to discuss some details in the test method employed that seem to be of importance for the homogeneity of the material and the accuracy of the results. The coleoptiles were selected between the length limits 2.5 and 3.0 cm. The cutting of the coleoptiles must be made with great care. The initial length of the sections must be very constant and the cut ends even. By means of a special cutting tool with two parallel razor blades the variation in the initial length was restricted to a few hundredth parts of a millimetre. A good contact between the sections and both the test solution and the air seems to be of great importance. The method of pressing the sections down in the solution but not under the surface by means of thin glass needles seems to meet this requirement very well. The measurements must be made after a certain rule. It is not always possible to obtain quite flat section ends. Sometimes small fringes arise that will affect the measurements to a small extent. The measurement always represented the greatest length with the fringes included.

Results

In table 1 is given the growth of coleoptiles, untreated and treated with $10^{-5} M$ PCIB. The tests were made on four occasions with one control and one treatment each time. The measurements are summarized (80 measurements for the control and 80 for the treatment) in the table. It is seen that PCIB lowers the growth of the coleoptiles and that the *t*-value shows good significance.

From table 2 is evident that the content of extractable IAA in the coleoptiles is higher when they are treated with $10^{-5} M$ PCIB than when they are untreated. The untreated ones, on the other hand, show a certain amount of IAA. Every measurement made is given in the table and no single value has been omitted. The experiments were made at five different times, each time with a test for the control of *Avena* section growth, one test with extract from untreated and one with extract from treated coleoptiles. In the fourth

Table 1. Growth of isolated *Avena* coleoptiles with tips intact treated at the base with *p*-chlorophenoxyisobutyric acid (PCIB). The figures represent the summarized numbers of coleoptiles from four experiments, each with 20 coleoptiles per treatment. Initial coleoptile length 20.0 mm. The difference between the means, 0.5 mm, has a *t*-value of 6.77** (3 degrees of freedom).

Conc. PCIB	Length in mm										Average length mm
	23.5	24.0	24.5	25.0	25.5	26.0	26.5	27.0	27.5	28.0	
0	4	1	4	13	10	14	15	14	2	3	25.9
$10^{-5} M$	2	7	12	14	11	23	8	2	0	1	25.4

Table 2. The IAA content of coleoptile extract expressed as growth of *Avena coleoptile* sections. The extracts are obtained from coleoptiles untreated and treated with 10^{-5} M p-chlorophenoxyisobutyric acid (PCIB). The figures represent numbers of sections. Each experiment comprises 10 sections per treatment. Initial section length 5.0 mm.

Treatment	Date of expt.	Section length in mm										Average growth mm
		5.3	5.4	5.5	5.6	5.7	5.8	5.9	6.0	6.1	6.2	
I Test control (no extract)	1.10.54		2	7			1					0.51
	3.10.54	2		6	1	1						0.49
	6.10.54	1	1	4	2		2					0.55
	2.11.54			1	1	4	3		1			0.73
	6.12.54	1	2	1	6							0.52
	Total	4	5	19	10	5	6	0	1			0.56
II With extract from untreated coleoptiles	1.10.54		1	2	3	2	2					0.62
	3.10.54		1	2	3	2	2					0.62
	6.10.54			3	2	4		1				0.64
	2.11.54					1	4	1	4			0.88
	6.12.54			2	3	4	1					0.64
	Total		2	9	11	13	9	2	4			0.68
III With extract from coleoptiles treated with 10^{-5} M PCIB	1.10.54			1	3	2	3		1			0.71
	3.10.54			1	2	2	3	1	1			0.74
	6.10.54			1		3	1	3	1			0.80
	2.11.54					2	3	1	1		3	0.93
	6.12.54					3	2			1		0.72
	Total			3	9	12	12	6	4	1	3	0.78

experiment the control growth diverges from the growth of the other controls but even in this case growth, when extract is supplied, exceeds that of the control. The deviating growth in the fourth experiment must be due to some casual variation in an unknown factor.

Statistical analysis

The authors are very grateful to Dr. Bertil Matérn, Forest Research Institute, Stockholm, for his working out the statistical analysis of the material given in table 2. The analysis is represented in table 3. Each experiment comprises three treatments and the analysis of variance has been carried out on the 15 observed values of average growth (the right column in table 2). The following variance ratios are obtained:

$$\frac{0.02935}{0.00050} = 58.7^{***} \text{ for experiments and } \frac{0.06067}{0.00050} = 121.3^{***} \text{ for treatments.}$$

Thus this analysis shows a significant difference both between experiments and treatments. In the case of the experiments this significance is due to the

Table 3. *Statistical analysis of the average growth values given in table 2.*

	Sum of squares	Degrees of freedom	Mean squares
Between experiments	0.11740	4	0.02935
Between treatments	0.12133	2	0.06067
Error	0.00400	8	0.00050
Total	0.23273	14	

deviating growth in the fourth experiment. Between the four others there is no significant difference ($p > 0.5$). The standard error of the difference between two treatment means is 0.014 and thus the difference between treatments I—II gives 0.12 ± 0.014 and between II—III 0.10 ± 0.014 .

Discussion

The test method has already been described. Every measurement made has been reported and the statistical analysis has shown that the reliability of the results is beyond doubt.

The results show that untreated coleoptiles contain a certain amount of extractable IAA and that this amount increases upon a treatment with PCIB. From this can be concluded that the effect of PCIB consists either in a liberation of IAA from some chemical complex or in preventing some IAA supplied by the tip from being consumed or bound in a non-extractable form. Two possibilities arise: that PCIB has a »sparing action» (cf. Henderson and Deese, 2, on the influence of 2,4-D on the IAA content of coleoptiles), for example by blocking the IAA oxidase, or that PCIB competes with IAA in some other reaction. Against the first possibility is the decrease in growth, which does not indicate such a sparing action. The second case is supported by the fact that PCIB has been shown to interact competitively with IAA.

The site of competition must be one where IAA exerts its growth activity, or PCIB should not decrease the coleoptile growth. This result seems to form more direct evidence that PCIB and IAA compete at the point of IAA action. From the results it is further obvious that the amount of extractable IAA is no measure of the amount of auxin active to promote growth when the antagonistic substance PCIB is present. It is, therefore, stressed that determinations of extractable IAA must be interpreted with great caution as far as growth activity is concerned.

Further investigations are being conducted on the subject.

Summary

The amount of short-time extractable IAA in *Avena* coleoptiles is measured after treatment with 10^{-5} M p-chlorophenoxyisobutyric acid (PCIB). It was found that:

1. Treatment with PCIB decreases the growth of the coleoptiles.
2. Treatment with PCIB increases the content of extractable IAA.

From the experimental data presented it is concluded that this effect of PCIB depends on a competition of PCIB and IAA at the point of IAA action.

On account of the results measurements of the content of extractable IAA in *Avena* coleoptiles must be interpreted with great caution as far as growth activity is concerned.

References

1. Burström, H.: Studies on growth and metabolism of roots. IV. Positive and negative auxin effects on cell elongation. — *Physiol. Plant.* 3: 277. 1950.
2. Henderson, J. H. M. & Deese, D. C.: Correlation between endogenous auxin and its destruction in vivo by 2,4-dichlorophenoxyacetic acid in plants. — *Nature* 174: 967. 1954.
3. Ingestad, T.: Kinetic aspects on the growth-regulating effect of some phenoxy acids. — *Physiol. Plant.* 6: 796. 1953.
4. McRae, D. H. & Bonner, J.: Diortho-substituted phenoxyacetic acids as antiauxins. — *Plant Physiol.* 27: 834. 1952.
5. — — Chemical structure and antiauxin activity. — *Physiol. Plant.* 6: 485. 1953.

On the Separation of Acidic and Non-Acidic Auxins

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In studying the various forms of auxin present in plant extracts it is often necessary to separate the acidic and the non-acidic components of the extracts. This is frequently done by shaking an ether extract with an alkaline, aqueous solution, which takes up the acid auxin, leaving the non-acidic substances in the ether. After separation of the two phases and acidification of the aqueous solution, the acid auxin can be shaken out with ether.

Sodium bicarbonate is used most frequently as the alkaline component of the aqueous solution. Various acids have been used for neutralizing the bicarbonate and acidifying the solution. Because of the acid-sensitivity of indole-3-acetic acid (IAA), the pH-value of the solution should be kept above a certain limit during the shaking with ether; and the acid used should be very sparingly soluble in ether since otherwise its concentration would become too high when the ether is subsequently condensed. The ether-solubility of acetic and citric acids, which have been used in the fractionation of auxin-containing extracts, is by far too high; and hydrochloric acid is easily overdosed. Boysen Jensen (1941) recommended tartaric acid, which is sparingly soluble in ether and is an excellent buffer around pH 3. Tartaric acid has been used by a great number of workers including the writer. During a study of auxins extracted from pollen grains, however, the writer found that ether shaken with a solution of tartaric acid contained materials which were inhibitory in the *Avena* coleoptile curvature test. This finding was briefly reported in a previous paper (Larsen and Tung, 1950). It will be shown below that tartaric acid will yield satisfactory results under certain circumstances. As a general

procedure, however, careful titration of the bicarbonate solution with hydrochloric acid to a pH-value of 2.7 to 2.8 is more reliable.

Auxin Tests

Auxin was determined in the *Avena* coleoptile curvature test using soil-grown test plants (Boysen Jensen, 1941) which were decapitated twice. Auxin was transferred to agar by a modification of Boysen Jensen's ether-dropping method. This modification and other details of the test have been fully described elsewhere (Larsen 1955). The agar platelets had a final concentration of 1.25 per cent agar (shredded USP agar), 0.005 *M* Sørensen's citrate buffer, and 0.001 *M* CaCl_2 . The pH-value of the undiluted (0.1 *M*) buffer solution was 6.00, and that of the final agar mixture was 6.3–6.4, measured electrometrically.

Partition of Indoleacetic Acid at Various pH-Values

Procedure: 5.0 mg. of IAA were dissolved in 50 ml. of peroxide-free ether from which water had been frozen out. Serial dilutions were made in ether. A total of 0.25 μg . of IAA dissolved in 12.5 ml. of ether was shaken three times in a separatory funnel, each time with 8.0 ml. water+2.0 ml. 0.50 *M* NaHCO_3 solution (pH=8.6). The addition of glucose to the aqueous phase (cf. Boysen Jensen) was omitted. Each shaking lasted for 3–4 min. The aqueous fractions were combined. In each experiment two 10-ml. portions of this solution, each containing 0.083 μg . of IAA and 1.0 millimol of NaHCO_3 , were acidified with different quantities of 1.0 *M* tartaric acid or 1.0 *N* HCl solution. The pH-value reached with various quantities of the acids can be read from the titration curves *A* and *B* in Figure 1. For instance, the addition of 1.0 ml. 1.0 *M* tartaric acid results in a pH-value of 3.55; and 1.07 ml. 1.0 *N* hydrochloric acid gives a pH-value of 2.8. After addition of the desired amount of acid, the aqueous solution was shaken with 3×9 ml. of ether. The three ether fractions were combined and their volume was adjusted to 25 ml. Of this solution, 0.9 ml. were evaporated on a 0.1-ml. agar platelet. If all auxin is recovered, the 0.9 ml. contain 0.0030 μg . of IAA. Theoretically, the maximum concentration of IAA in the agar platelet is thus 30 $\mu\text{g}/\text{l}$. The actual concentration was read from a standard activity curve and expressed as percentages of 30 $\mu\text{g}/\text{l}$.

The theoretical recovery of IAA in three shakings was computed on the basis of the following data:

- (1) The pK-value for IAA at 20° C.: 4.65 (mean of values quoted by van Overbeek et al., 1951). — (2) The pH-value of the aqueous solution. The ratio *A* of dissociated to undissociated IAA was computed from (1) and (2): $\log A = \text{pH} - \text{pK}$. — (3) The partition coefficient for undissociated IAA distributed between wet ether and ether-saturated water: 9.4 (Dolk and Thimann, 1932). This value actually refers to +1° C., but no correction was made for temperature. — (4) The mutual solubilities of water and ether: 3 ml. water in 100 ml. ether, and 8 ml. ether in 100 ml. water; thus 9 ml. of ether + e.g. 11 ml. of water will make 8.3 ml. of wet ether + 11.7 ml. of ether-saturated water. The corrections computed for the first shaking were applied unchanged to the second and third. — The calculations were made as follows (compare Table 1).

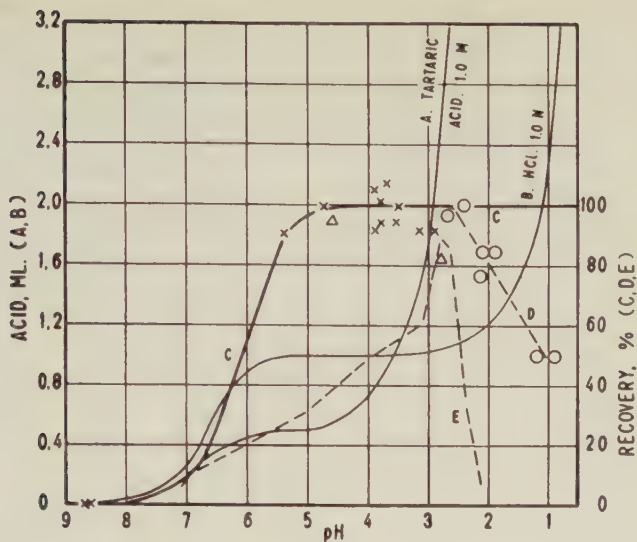


Figure 1. *Partition experiments.* — Curves A and B: pH-values of the aqueous phase (10 ml. 0.10 M NaHCO_3) during successive stages of titration with 1.0 M tartaric or 1.0 N hydrochloric acid. Volumes of acid indicated at the left-hand ordinate.

Curve C: Theoretical recovery of IAA in three shakings at the pH-values indicated. Data from Table 1. *Right-hand ordinate.* — Crosses: Experimental recovery of 0.083 μg . of IAA dissolved in 8 ml. water + 2.0 ml. 0.50 M NaHCO_3 . Acidification with 1.0 M tartaric acid. — Curve D (Circles): As above, except that acidification was done with 1.0 N HCl. — Triangles: Recovery of IAA and auxin extracted from Avena coleoptiles. Aqueous phase buffered with Na_2HPO_4 and citric acid. Recoveries expressed as percentages of curvature. Data from Terpstra (1953, Table 1). — Curve E: Recovery of 0.1 μg . of IAA from solutions of HCl or NaOH (cf. text). Data from Gordon and Sánchez Nieva (1949, Fig. 1).

The quantities of IAA in the system may be termed a (=dissociated molecules in the aqueous phase), b (=undissociated molecules in the aqueous phase), and c (=IAA in the ether). It is assumed that only undissociated molecules are soluble in ether, although it is possible that wet ether may contain a small fraction of dissociated IAA. At equilibrium after the first shaking we have: (1) $\log \frac{a}{b} = \text{pH} - \text{pK}$;

$\frac{a}{b} = A$; $a = bA$. (2) $\frac{c}{b} = 9.4 \times \frac{\text{volume of ether}}{\text{volume of water}} = B$; $c = bB$; (3) Fraction in ether =

$\frac{c}{a+b+c} = \frac{bB}{bA+b+bB} = \frac{B}{A+1+B}$. For convenience in the subsequent calculations this

expression is converted to $\frac{B}{1+A}$ in which $\frac{B}{1+A}$ may be termed C . The fractions

Table 1. *Computation of the theoretical recovery of IAA in three subsequent partitions between ether and water at various pH-values.*

pH	A = ratio diss. IAA	Volume of aqueous phase, ml.	B = ratio IAA in ether	C = B 1 + A	Fraction of IAA present in the ether phase in each of three subsequent partitions, %			Theoret- ical reco- very, % F ₁ +F ₂ +F ₃	
	undiss. IAA in aqueous phase. ¹		undiss. IAA in aq.		= 9.4 · Vol. ether Vol. aq. (Vol. ether=8.3 ml)	F ₁ = 100 C	F ₂ = 100 C		F ₃ = 100 C
							1 + C		(1 + C) ²
2.65	0.01	11.80	6.61	6.54	86.74	11.50	1.53	99.8	
3.65	0.1	11.60	6.73	6.12	85.96	12.07	1.70	99.7	
4.65	1.	11.20	6.97	3.48	77.68	17.34	3.87	98.9	
5.00	2.239	11.20	6.97	2.15	68.25	21.67	6.88	96.8	
5.35	5.012	11.20	6.97	1.159	53.68	24.86	11.52	90.1	
5.65	10.	11.20	6.97	0.634	38.80	23.75	14.53	77.1	
6.00	22.39	11.16	6.99	0.2988	23.01	17.71	13.64	54.4	
6.65	100	11.00	7.09	0.0702	6.56	6.13	5.73	18.4	
7.00	223.9	10.85	7.19	0.03197	3.10	3.00	2.91	9.0	
7.65	10 ³	10.70	7.29	0.00728	0.72	0.72	0.71	2.2	
8.65	10 ⁴	10.70	7.29	0.00073	0.07	0.07	0.07	0.2	

¹ From log A = pH — pK, in which pK = 4.65.

of the total amount of IAA present in the ether phase in each of three subsequent shakings are then $f_1 = \frac{C}{1+C}$; $f_2 = \frac{C}{(1+C)^2}$; $f_3 = \frac{C}{(1+C)^3}$.

The various steps in the computations are shown in Table 1; and the percentage of IAA present in the combined ether fractions is plotted in Figure 1, Curve C. This curve shows the efficiency of three shakings with ether. For instance, at pH = 5.35 only 16.63 per cent of the IAA is in the undissociated state. One shaking with ether (11.20 ml. ether-saturated water + 8.30 ml. wet ether), however, removes 53.7 per cent of the total amount of IAA. The second and third shakings remove 24.9 and 11.5 per cent, respectively. Three shakings thus remove 90.1 per cent of all IAA in the aqueous solution. The explanation of this high yield lies in the fact that removal of undissociated IAA from the aqueous phase leads to association of some of the dissociated molecules, which are thereby converted to the ether-soluble form.

It is evident from Table 1 that even at a favorable pH-value, such as 2.65, more than one shaking is needed to remove a quantity of IAA which is sufficiently close to 100 per cent. On the other hand, even at pH 5.0, three shakings will remove 96.8 per cent of the IAA. The data of Table 1 may also have some bearing on the importance of adjusting the pH-value of the aqueous phase in plant material from which it is desired to extract auxin with ether. If the pH-value is too high, even a large number of changes of ether will not remove all of the auxin. In extraction work, however, the ratio of

ether to water is generally more favorable than in the examples shown in the table.

The recovery IAA obtained in three shakings at various pH-values is shown in Figure 1 in which the crosses and circles represent experiments with tartaric and with hydrochloric acid, respectively. Over the range of pH-values from 8.7 to 2.4 the points are fairly close to the theoretical yield (Curve *C*). When the pH-value is reduced below 2.4, however, there is a steady drop in recovery (Curve *D*). The triangles (Terpstra, 1953) indicate recoveries from solutions buffered with Na_2HPO_4 and citric acid. These values are also in fairly good agreement with expectation. Curve *E* represents recoveries found by Gordon and Sánchez Nieva (1949) in experiments in which 0.1 μg . of IAA in 25 ml. of water was partitioned 3 times with 3 volumes of ether after the pH-value of the aqueous phase had been adjusted with HCl or NaOH. Except at pH 2.6 and 2.8 the recoveries are considerably lower than the theoretical values (which are higher than indicated by Curve *C* in Figure 1 since the ratio between ether and water was 3 to 1). Part of the explanation of the course of the recovery curve found by Gordon and Sánchez Nieva may be sought in the fact that the acidity in solutions of strong acids is very poorly defined at pH-values higher than ca 3.5.

It is evident from Figure 1 that an inactivation of IAA occurs at pH-values lower than ca. 2.5. Partition should, therefore, be carried out at somewhat higher pH-values; and the same precaution should be taken in extractions of auxin from plant material.

Choice of Acid for Adjusting the Acidity

On the basis of the results shown in Figure 1 it seemed natural to use tartaric acid for adjusting the acidity of the aqueous phase and carry out partitions at pH-values between 3.5 and 4. This procedure yielded excellent results with about 0.08 μg . of IAA or more in the original ether. With less than 0.08 μg . in the original ether solution, however, a 100 per cent recovery was never obtained. As mentioned above, the final solution of IAA in ether was made up to 25 ml. When 0.08 μg . of IAA was present in this volume, less than one ml. was required for one auxin determination. With decreasing total amounts of IAA, the volume needed for one determination had to be increased, but at the same time the percentage of IAA recovered seemed to become lower and lower. Such results indicated that the final ether solution might contain a constituent which reduced the curvature of the test plants. This constituent might be tartaric acid or it might be some impurity contained in the chemicals (NaHCO_3 or tartaric acid). These possibilities were investigated in a series of partition experiments carried out as above, but without

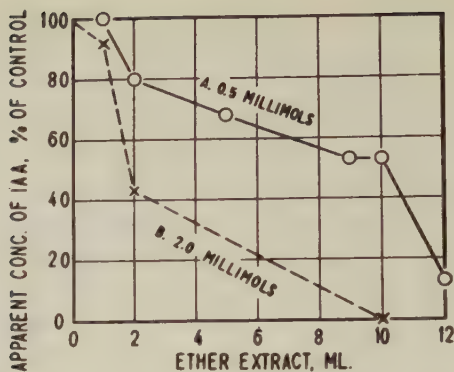


Figure 2. *Inhibitory effect of ether extracts of tartaric acid.* 11 ml. of an aqueous solution, containing 0.95 millimols of NaHCO_3 and the desired amount of acid, were shaken with three 9-ml. portions of ether which were then combined and adjusted to 25 ml. — *Curve A:* 0.5 millimols of tartaric acid, $\text{pH}=4.7$. — *Curve B:* 2.0 millimols of tartaric acid, $\text{pH}=2.9$. — *Abscissa:* Number of ml. of auxin-free ether extract evaporated on 0.1-ml. agar platelets together with 0.0040 μg . of IAA dissolved in 4 ml. ether. — *Ordinate:* Apparent concentration of IAA expressed as percentages of the control value, 40 $\mu\text{g}/\text{l}$.

IAA. Various quantities of the final, auxin-free ether solution were evaporated on 0.1-ml. agar platelets together with a solution of 0.0040 μg . of IAA in 4 ml. ether. The resulting curvatures were converted to concentrations of IAA and expressed as percentages of 40 $\mu\text{g}/\text{l}$.

The bicarbonate proved to be harmless. Ten ml. of ether were shaken with 3 successive portions of 8 ml. water+2 ml. 0.5 M NaHCO_3 ($\text{pH}=8.6$). The ether volume was readjusted to 10 ml. and various amounts tested as described. The concentration of IAA found with 3 ml. of 'bicarbonate extract' was 102 per cent of the control, and with 7 ml. it was 95, 95, and 105 per cent in three experiments. These determinations also show that the ether itself, at least in the quantities used, is without any effect on the response of the test plants.

Ether shaken with bicarbonate solutions which had been acidified with tartaric acid, on the other hand, reduced the curvatures of the test plants. Figure 2 shows that the apparent concentration of IAA in the agar becomes lower and lower when increasing amounts of 'acid ether' are evaporated on the platelets. Essentially similar results were obtained with natural auxins (extracted from maize endosperm) by Mr. Edwin Alder, working in this laboratory (unpublished).

These results might be explained by the fact that tartaric acid is somewhat soluble in ether. Increasing amounts of tartaric acid transferred to the agar platelets would lower the pH -value of the agar which might thereby become too acid for maximal response of the test plants to a given concentration of IAA.

The reduction of curvature might, however, also have been caused by impurities in the solution of tartaric acid. The particular solution which was used for the experiments shown in Figure 2 had been stored at $+5^\circ \text{C}$. for

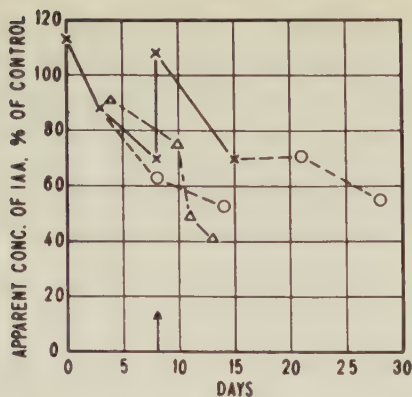


Figure 3. Formation of the inhibitory factor in solutions of tartaric acid. Same general procedure as described in legend to Fig. 2. — Crosses and circles: 1.9 millimols of NaHCO_3 + 2.0 millimols of tartaric acid. pH 3.5. 5 ml. ether tested each time. — Crosses: An aqueous solution of tartaric acid (1.0 *M*) made up, tested at zero time, stored at $+5^\circ \text{C}$., and re-tested at intervals. Increasing inhibition with aging. Three ether-shakings of the eight-day-old solution (arrow) remove the inhibitor. Inhibitor present again after 7 more days. — Circles: Ether fractions tested on the 1st, 3rd, and 15th day were stored at $+5^\circ \text{C}$. and re-tested at intervals. Concentration of inhibitor increases. — Triangles: 34 ml. of an aqueous solution containing 11.4 millimols of NaHCO_3 + 12.0 millimols of tartaric acid. pH 3.5. Shaken with four 26-ml. portions of ether. Ether adjusted to 100 ml. and stored at $+5^\circ \text{C}$. 8 ml. ether tested each time. Increasing inhibition with aging.

several weeks. Ten ml. of this solution (conc.: 1.0 *M*) were shaken with three or six 10-ml. portions of ether. The ether fractions were discarded, and the purified tartaric acid was used for acidifying bicarbonate solutions to pH 3.5. These solutions were shaken with three 9-ml. portions of ether in the usual manner, and the resulting »acid ether» (25 ml) was tested for inhibitory activity against IAA (40 $\mu\text{g./l}$). It was found that the inhibitory effect of tartaric acid could be reduced by shaking the solution with ether before use. The results of a similar experiment are included in Figure 3. These results indicate that only part of the inhibitory activity of the ether-extract may be due to dissolved tartaric acid. A considerable part of the inhibitory activity seems to stem from impurities which can be removed by shaking with ether.

A series of experiments were carried out in order to study the effect of storage on the formation of the inhibitor(s). In Figure 3 the full lines represent experiments with tartaric acid which had been stored at $+5^\circ \text{C}$. in aqueous solution (1 *M*) for various periods of time before it was used for acidifying bicarbonate solutions to pH 3.5. The figure shows that the growth-retarding effect increases gradually during the first 8 days after the solution was made up. On the 8th day, the solution was purified by three shakings with ether

before use. This treatment removed the growth-retarding factor, but after 7 additional days at $+5^{\circ}$ C. the solution had the same inhibitory effect as just before the purification. The broken lines in Figure 3 represent the results of experiments in which the ether solutions obtained by shaking the acidified solutions (at pH 3.5) were tested for inhibitor after storage at $+5^{\circ}$ C. for various periods of time. These experiments show that the inhibitory effect of such ether solutions increases during storage at $+5^{\circ}$ C.

The solutions of tartaric acid were not sterilized, so the possibility existed that microorganisms were responsible for the formation of the inhibitor in aqueous solutions. This is of course excluded as far as the solutions in ether are concerned. It is not very likely, however, that a sufficiently active microflora should develop in aqueous solutions having a pH-value of 1.5 and stored at $+5^{\circ}$ C. for only 3 days, after which a distinct inhibition could be demonstrated. Fungal mycelia had developed in a 1.0 *M* solution of tartaric acid which had been stored at $+5^{\circ}$ C. for 7 $\frac{1}{2}$ months. This solution proved to contain auxin, about 5×10^{-3} μ g. per ml., computed as IAA. (Since this auxin is stable at pH 1.5, it may not be IAA, but no attempt was made to establish its chemical nature.) This result does not support the assumption of a microbial formation of inhibitor. The following experiment shows beyond doubt that the formation of the inhibitor can take place without the participation of microorganisms.

Two millimols (=300 mg.) of crystalline D-tartaric acid were weighed out and added to a solution of 1.90 millimols of NaHCO_3 in 10 ml. of water (pH 3.5). The mixture was shaken out immediately with 3×9 ml. of ether. The volume of ether was adjusted to 25 ml.; and 5 ml. were tested together with IAA, 40 μ g./l. The apparent concentration was 69 per cent of the control. When 5 ml. of the same ether solution were tested the following day, the apparent concentration of IAA was 60 per cent. Thus 31 per cent and 40 per cent inhibition were found in spite of the fact that the tartaric acid was present in aqueous solution for only about 15 minutes (namely during the shaking) before the inhibitor was removed. A repetition of the experiment yielded similar results. It is unknown why the inhibitor can be detected sooner after the addition of crystals than after addition of the same amount of acid in the form of an aqueous solution.

In contrast to the above-mentioned results no inhibitory factor was present in the ether when hydrochloric acid was used for acidifying the bicarbonate solution. Examples: (1) 6.2 ml. water + 3.8 ml. 0.50 *M* NaHCO_3 + 2.0 ml. 1.0 *N* HCl (pH 2.2), were shaken with three 9-ml. portions of ether. The volume of the resulting «acid ether» was adjusted to 25 ml.; and 12 ml. were evaporated on a 0.1-ml. agar platelet containing 40 μ g. of IAA per l. The resulting curvatures corresponded to 95 per cent of the theoretical concentration. (2) 8.1 ml. water + 1.9 ml. 0.50 *M* NaHCO_3 + 1.15 ml. 1.0 *N* HCl (pH 1.9) treated as in (1). The concentration of IAA found with 5 ml. of the «acid ether» was 110 per cent of the control value.

In the two examples the solutions were made considerably more acid than

required for a satisfactory recovery of IAA in the partition experiments. In spite hereof, no inhibitory effect was observed in ether extracts of the acidified solutions. As a consequence, hydrochloric acid must be considered suitable for acidifying the bicarbonate solution if a sufficiently stable acidity could be maintained in the region in which IAA is stable, i.e. at pH-values above ca. 2.4. As seen from the titration curve *B* in Figure 1 this leaves the narrow region between pH 2.4 and pH 3.0, which is traversed by less than 0.1 ml. of 1.0 *N* HCl solution. Under these circumstances the accuracy in volumetric measurement of the solutions of bicarbonate and acid is hardly sufficient. The accuracy might be increased by diluting the acid, but since all the bicarbonate will have to be neutralized, any considerable dilution of the acid would result in large volumes of aqueous solution, which would require large volumes of ether in the subsequent partition. Any increase in the accuracy of measuring, however, is offset by the fact that ether extracts of plant material may contain an unknown quantity of organic acid. The only reliable procedure is to control the acidity of the acidified solution either by electrometric measurements or by means of an indicator. Figure 1 shows the results of partitions in which the pH-value was measured electrometrically. Terpstra (1953) recommended the use of methyl orange as an indicator. On the basis of this recommendation the following standard procedure was worked out.

The volume of the ether extract is adjusted to 10 or 15 ml., and the extract is shaken with three portions of 7 ml. water + 2.0 ml. 0.50 *M* NaHCO₃ solution. The aqueous fractions are combined and titrated carefully with a 0.5 *N* HCl solution, using 5 drops of a 0.02 per cent aqueous solution of methyl orange as an indicator. After the color of the solution has changed from yellow through orange to clear red (pH 3.5), two more drops of the acid should be added. During the color change the solution should be shaken with special care to drive off all carbon dioxide. When working with solutions of pure IAA, approximately 6.30 ml. 0.50 *N* HCl solution is required to reach a pH-value of 2.75. The aqueous solution is now shaken with 3 × 18 ml. of ether. The ether fractions are combined and evaporated to 25 ml. which constitute the acidic fraction of the ether extract.

In control experiments by Terpstra (1953) and by Mr. Edwin Alder in the present laboratory (unpublished) the indicator remained in the aqueous phase and proved to be without any influence on the response of the test plants. — It might be possible to select an indicator the effective range of which agreed more closely with the usable range of pH-values (2.5–3.0). The indicator should be soluble in water without the addition of alcohol, and its effect on the curvatures of *Avena* test plants should be negligible.

Quantities of Acid Transferred to the Agar

The results of the experiments on storage and purification of solutions of tartaric acid seemed to indicate that the growth-retarding effect of ether extracts of such solutions was not due exclusively to the small amounts of tartaric acid which passed into the ether. A special study, however, was made for the purpose of estimating the actual content of tartaric acid and HCl in the various ether solutions. In these experiments the ether was evaporated, the residue dissolved in 10 ml. of water, and titrated with 0.01 *N* NaOH, using phenolphthalein as an indicator. Since 100 ml. distilled water required 0.90 ml. of 0.01 *N* NaOH for neutralization, a blind value of 0.09 ml. was subtracted from all the titration values.

Solubility of tartaric acid in wet ether. Twenty ml. of water-saturated ether was poured on various quantities of crystalline tartaric acid. The suspensions were shaken occasionally and left for 24 hours. The ether was decanted off, and 1-ml. portions were titrated. The following quantities of tartaric acid were used: 0.33 g., 0.80 g. and 1.20 g. The corresponding concentrations of acid in the ether were 5.31, 5.33 and 5.68 millimols per 100 ml., respectively. If the highest value is taken, this corresponds to 852 mg. of D-tartaric acid in 100 ml. of ether. In the Handbook of Chemistry and Physics (1954, p. 1165) the solubilities of DL- and D-tartaric acids are given as 0.87 and 0.44 g., respectively, in 100 ml. of ether at room temperature. It was presumed that these values referred to dry ether, and that water-saturated ether might dissolve more. When a saturated solution of tartaric acid was kept at minus 25° C. for 24 hrs., the major part of the water was frozen out. The concentration of tartaric acid in the ether, however, remained unchanged.

The coefficient of partition of tartaric acid between wet ether and ether-saturated water was determined by partitioning 5 or 10 millimols of the acid between 15 ml. of water and 25 ml. of ether. The two dissociation constants of tartaric acid are (at 18° C.): $K_1 = 9.6 \times 10^{-4}$ and $K_2 = 2.8 \times 10^{-5}$; $pK_1 = 3.02$ $pK_2 = 4.55$ (Kolthoff 1932 p. 389). The second dissociation constant could be neglected in the computation of the acidity. The pH-values of the two solutions were 1.73 and 1.58, respectively (calculated). At these pH-values the fractions of undissociated tartaric acid are 94.5 per cent and 96.0 per cent, respectively. At equilibrium, the concentration of tartaric acid in the ether phase may be called *a*, and in the water phase *b*; the partition coefficient thus equals $C = \frac{a}{b \times \text{undiss. fraction}}$. The mean of the *C*-values was 3.75×10^{-3} . On the basis of this value and the first dissociation constant of tartaric acid the concentration of acid in the ether in the various partition experiments was computed. Some of the computed values were verified by titration (Table 2).

Solubility of hydrochloric acid in wet ether. A mixture of 1 ml. concentrated HCl-solution and 30 ml. water-saturated ether was shaken occasionally and left for 24 hrs. The ether was decanted off, and 1- and 2-ml. portions were titrated. The concentration of acid in the ether was 1.28 millimols per 100 ml. This experiment shows that wet ether does dissolve HCl. Whether the acid is present in dissociated form in the water dissolved in the ether, or for instance as dissolved, undissociated gas, is of minor importance in the present connection.

Table 2. *Content of tartaric acid in the combined ether fractions after three partitions at different pH-values. Comparison between computed amounts and amounts determined directly by titration.*

Amt. of NaHCO ₃ , millimols	Amt. of tartaric acid, millimols	Volume of aq. phase, ml.	pH of aq. phase	Volume of ether for each of 3 shakings, ml.	Total content of acid in combined ether fractions, micromols	
					Computed	Titrated
0.95	2.00	11.7	2.9	8.3	7.75	7.6
3.00	3.00	32.1	3.6	15.9	3.12	3.25
3.00	1.58	32.1	4.7	15.9	0.16	< 0.2

The coefficient of partition of HCl between wet ether and ether-saturated water was determined in the same manner as the partition coefficient of tartaric acid, with the exception that the computations were based on the total concentration of HCl, assuming that no undissociated fraction was present. The partition coefficient of HCl was found to be of the order of 3×10^{-4} , i.e. less than one tenth of that of tartaric acid. By using this value the quantities of HCl in the ether fractions obtained in the various partition experiments were computed. The computations were based on the excess of HCl present in the aqueous phase after neutralization of the bicarbonate. The actual quantities of HCl in the ether fractions were so small that they could not be determined with the titration technique which was used for tartaric acid. The titration values with 0.01 *N* NaOH were of the same order as the blind value (0.09 ml.). No attempt was made to make accurate determinations, since this result clearly indicated that the content of HCl in the ether was lower than 3×10^{-4} millimols in 25. ml.

Acidity of the agar. The test agar was buffered with Sørensen's citrate buffer: 58.7 ml. 0.1 *M* citrate plus 41.3 ml. 0.1 *M* NaOH solution. The pH-value of this mixture is 6.0. In the agar, the concentration of the buffer is 0.005 *M*; and CaCl₂ is present in a concentration of 0.001 *M*. The pH-value of the mixture at this dilution is 6.43. Tests were carried out with 0.1-ml. agar platelets on which various volumes of ether solution had been evaporated. The quantities of tartaric or hydrochloric acid transferred to the agar in the various partition experiments were computed from the data given in the preceding paragraphs. The concentration of acid in the agar can thus be computed. (Table 3) Solutions having the same concentrations of buffer and acid as those computed for the agar were made up; and their pH-values were measured electrometrically. These values are entered in Table 3 as pH of agar.

In Table 3 the inhibition of the response of the test plants is expressed as the difference, in percentages, between the actual and the apparent concentration of IAA. This value is compared with various other data concerning the partition experiments.

It is evident that the quantities of hydrochloric acid which are transferred to the agar are quite small and have little influence on the acidity of the agar, even when partition took place at the extreme pH-values of 1.9 and 2.2. The small changes in the pH-values of the agar do not seem to have any measur-

Table 3. Partition of tartaric and hydrochloric acids between ether and water at various pH-values. Concentration of acid in the combined ether fractions, which were adjusted to 25 ml. Influence of various amounts of »acid ether» on the pH-value of the agar and the response of *Avena test coleoptiles*.

Acid used	Amt. of NaHCO ₃ , millimols	Total amt. of acid added, millimols	pH of aq. phase	Conc. of acid in ether, millimols per ml.	Volume of ether tested, ml.	Conc. of tartaric acid or HCl in agar, molar.	pH of agar ³	P = product of total amt. of acid added and volume of ether tested	Inhibition of response, % IAA
HCl ¹	0.90	1.15	1.9	6.30×10^{-6}	5	3.15×10^{-4}	6.24		0 — 5
					25	1.58×10^{-3}	5.57		— ⁴
HCl ¹	1.85	2.00	2.2	3.52×10^{-6}	12	4.21×10^{-4}	6.17		0
HCl, standard procedure ²	3.00	3.15	2.75	2.59×10^{-6}	1	2.59×10^{-5}	6.42		0
					10	2.59×10^{-4}	6.25		0
					25	6.48×10^{-4}	6.05		0
Tartaric acid ¹	0.95	0.5	4.7	2.86×10^{-6}	1	2.86×10^{-5}	6.42	0.5	0
					2	5.72×10^{-5}	6.40	1.0	20
					5	1.43×10^{-4}	6.28	2.5	32
					9	2.58×10^{-4}	6.14	4.5	47
					10	2.86×10^{-4}	6.09	5.0	47
					12	3.44×10^{-4}	6.05	6.0	87
Tartaric acid ¹	0.95	2.0	2.9	3.10×10^{-4}	1	3.10×10^{-3}	4.09	2.0	8
					2	6.20×10^{-3}	3.43	4.0	57
					10	3.10×10^{-2}	2.60	20.0	100

¹ Volume of aqueous phase ca. 11 ml. Volume of ether for each of 3 shakings 9 ml.

² Volume of aqueous phase ca. 33 ml. Volume of ether for each of 3 shakings 18 ml.

³ Agar buffered with 0.005 M citrate buffer, pH=6.43.

⁴ Not tested.

able influence on the response of the test plants since no inhibition greater than 5 per cent was found.

Data for two series of experiments with tartaric acid are given in Table 3. In the first series, only 0.5 millimols of tartaric acid were present. Using this amount of acid, considerable inhibitions are observed, but the changes in the pH-value of the agar are of the same order of magnitude as those found with quantities of HCl which produced no inhibition. Already these results indicate that the inhibition caused by tartaric acid is not due to its influence on the acidity of the agar. In the second series (with a total of 2 millimols of acid) the changes in the pH-value of the agar are much greater, but the inhibitions at pH-values of 3.4 and 4.1 are smaller than the inhibition at pH 6.05 in the first series. The relationship between the pH-value of the agar and the amount of inhibition in the two series is plotted in Figure 4, curves A and B. It is evident from this figure that the amount of inhibition is not deter-

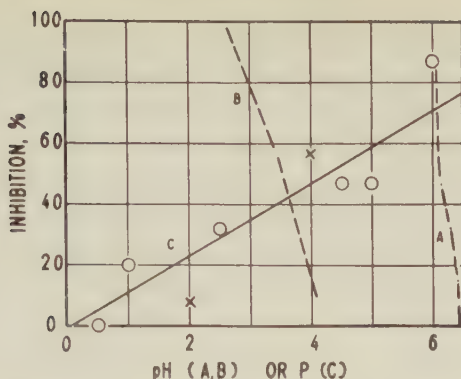


Figure 4. Inhibitory effect of ether extracts of mixtures of NaHCO_3 and tartaric acid. Compare Table 3. — Ordinate: Inhibition of response of test plants to IAA, 40 $\mu\text{g./l.}$ Difference, as percentages, between actual and apparent concentration. — Abscissa for Curves A and B: pH-value of agar after evaporation of ether extract on platelet. — Abscissa for Curve C: Product (P) of total amount of tartaric acid added to the aqueous phase and volume of ether extract tested.

mined by the acidity as such, since for instance 50 per cent inhibition can be obtained both at pH 6.05 and at pH 3.5.

A closer relationship seems to exist between the inhibition and the value P entered in Table 3. P is the product of the total amount of tartaric acid added to the aqueous phase and the volume of ether evaporated on the agar platelet. The inhibition is plotted against P in Figure 4, Curve C. In this plot the values from the two series of partition experiments can be visualized as belonging to one curve. This is impossible if inhibition is plotted against the pH-value or the concentration of tartaric acid in the agar. If the inhibition is independent of the latter and proportional to P , the partition characteristics of the inhibitor must be quite different from those of the free tartaric acid. The inhibitor can thus not be identical with free tartaric acid. The inhibitor was formed during storage in three different brands of analytical grade D-tartaric acid.

The principal purpose of the present study was to work out a reliable partition procedure. As a result, hydrochloric acid is recommended in preference to tartaric acid. The nature of the inhibitor formed in solutions of tartaric acid, however, was not investigated further.

Summary

1. The characteristics of partition of indole-3-acetic acid (IAA) at various pH-values were studied. Practically 100 per cent of the auxin can be recovered

in the ether fractions by three subsequent partitions between ether and water (in the proportion of 1 : 1 or 2 : 3) at pH-values ranging from 2.5 to 4.7. At pH-values lower than 2.5, IAA becomes inactivated.

2. A substance which reduces the response of *Avena* test plants to a given concentration of IAA is formed in solutions of tartaric acid during storage at $+5^{\circ}$ C. When such solutions are used for acidifying solutions of IAA and NaHCO_3 and these are shaken with ether, the inhibitor passes into the ether phase. The inhibitor is also formed in ether extracts of tartaric acid, but is not identical with this acid.

3. The formation of the growth inhibitor in solutions of tartaric acid does not preclude the use of this acid for acidifying the aqueous phase when the total amount of IAA present is 0.08 $\mu\text{g.}$ or higher. When smaller quantities of IAA are present, however, it is necessary to test larger volumes of the final ether solution; and thereby the concentration of inhibitor in the agar will become high enough to produce a noticeable reduction of the response of the test plants.

4. Hydrochloric acid is very sparingly soluble in ether and has a partition coefficient (ether/water) of the order of 3×10^{-4} . Ether shaken with 0.01 N solutions of HCl and evaporated on agar together with IAA had no inhibitory effect on the response of the test coleoptiles.

5. As a general procedure for the acidification of a solution of auxin and bicarbonate it is recommended to titrate the solution carefully with a 0.50 N solution of HCl, using a 0.02 per cent aqueous solution of methyl orange as an indicator (Terpstra, 1953). When the color of the indicator has changed from yellow through orange to clear red, two more drops of the acid should be added per 30 ml. aqueous solution (final pH 2.7—2.8). In subsequent partitions between ether and water, the indicator remains in the aqueous phase and is without any influence on the response of the test plants.

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References

- Boysen Jensen, P.: Quantitative Bestimmung der beschleunigenden Streckungswuchsstoffe in der sauren Fraktion der Ätherextrakte aus höheren Pflanzen. — *Planta* 31: 653. 1941.
- Dolk, H. E. & Thimann, K. V.: Studies on the growth hormone of plants. I. — *Proc. Natl. Acad. Sci. U.S.A.* 18: 30. 1932.

- Gordon, S. A. & Sánchez Nieva, F.: The biosynthesis of auxin in the vegetative pineapple. I. Nature of the active auxin. — *Arch. Biochem.* 20: 356. 1949.
- Kolthoff, I. M.: Säure-Basen-Indicatoren. — XII+416 pp. Berlin (Springer) 1932.
- Larsen, P.: Growth substances in higher plants. — In K. Paech and M. V. Tracey (ed.): »Modern Methods of Plant Analysis». Vol. III: 565. Heidelberg (Springer) 1955. (In press.)
- & Tung, S. M.: Growth-promoting and growth-retarding substances in pollen from diploid and triploid apple varieties. — *Bot. Gaz.* 111: 436. 1950.
- Terpstra, Willemke: Extraction and identification of growth substances. — 64 pp. Thesis, Utrecht, 1953.
- van Overbeek J., Blondeau, R. & Horne, Vesta: Trans-cinnamic acid as an anti-auxin. — *Am. Journ. Bot.* 38: 589. 1951.

**Studies on the Browning and Blackening of Plant Tissues.
III. Occurrence in the Leaves of Dahlia and Several Other
Plants of Chlorogenic Acid as the Principal
Browning Agent**

By

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Introduction

Leaves of *Dahlia variabilis* show remarkable death-ring (Todesring in the sense of Molisch) on heating with a lighted cigarette tip or other heated objects, and, as is well known, become apparently black when they are killed by the first sudden frost in early winter. This black color produced on a leaf is not in fact black, but is due to the presence in the cells of amorphous brown particles and green chloroplasts. It is very likely that also in this case, as is the case with *Stizolobium Hassjoo* (9), there must be a substance or substances responsible for this color change, that is, for the formation of brown color. We have been engaged in isolating the possible precursor of the brown substance, but there has been no hope of success yet in isolating it in a pure state. The aqueous or methanolic extract of the leaves of *Dahlia* gives a green color with ferric chloride, an indication that there might be an orthodihydroxyphenyl derivative which would be capable of changing into a brown substance by an oxidase. We submitted the four fractions of the leaf extract to paper-chromatographic analysis and found that chlorogenic acid and caffeic acid were present in every fraction, and as they were readily oxidized by leaf polyphenoloxidase in solution to become brown, these two acids may be regarded as the cause of the browning reaction of the leaves of *Dahlia*.

We have further examined *Aralia cordata*, *Clematis paniculata*, *Cryptotaenia japonica*, *Ilex latifolia*, *Inula salicina*, *Spiraea japonica* var. *ovatifolia*, and *Viburnum Sargentii*, and found the presence of chlogenic acid in all of them. Of these, caffeic acid was accompanied by chlorogenic acid in *Clematis* and *Viburnum*.

Experimental

1. Extraction and fractionation of *Dahlia* leaf constituents.

(Procedure A). One hundred grams of *Dahlia* leaves (a variety with white flowers) were picked and thrown into boiling water and boiled 1 hour. The extract was then filtered and the filtrate treated with aqueous lead acetate solution. The light brownish yellow precipitate was filtered, washed thoroughly with water and treated in a suspension in water with hydrogen sulfide. The precipitated lead sulfide was filtered off, the filtrate was decolorized with charcoal, and ethanol was added to the filtered solution. After filtering, the liquid was concentrated and exhaustively extracted with ether by shaking (ether extract II, aqueous solution I). The first filtrate of lead precipitate was treated with hydrogen sulfide, followed by filtering from lead sulfide and decolorizing with charcoal. The filtered colorless solution was concentrated (III).

These three fractions reacted with the polyphenoloxidase preparations obtained from *Dahlia* and tobacco leaves, becoming brown in a few minutes.

From each of these fractions ascending paper-chromatogram was run with a whole sheet of Whatman filter paper No. 54, solvent system butanol-acetic acid-water (4 : 1 : 2) being applied (Fig. 1).

On this paper-chromatogram there were found under ultraviolet light four clear zones: 1) pale yellow zone ($R_f=0.30-0.50$), 2) deep yellow zone ($R_f=0.57-0.65$), 3) bluish white zone ($R_f=0.72-0.80$), and 4) bluish white zone ($R_f=0.82-0.98$), which were then cut from one another. Zones with R_f values smaller than 0.30 were discarded because of its negative reaction with ferric chloride. These zones were separately extracted with boiling ethanol and the extracts were concentrated under diminished pressure to 0.5 ml. Of these concentrated extracts paper-chromatograms were made with Whatman filter paper No. 1 strip. The solvent systems used were butanol-acetic acid-water (4 : 1 : 2) and 80 per cent phenol, and the spots were treated with ultraviolet light, Hoepfner's reagent (sodium nitrite dissolved in 10 per cent acid to 1 per cent (10), and ethanolic ferric chloride solution.

As is shown in Figure 2, there were found several spots in each of the four chromatograms derived from these zones. Among them a spot of R_f 0.58 was common. This spot fluoresced bluish under ultraviolet light and yellow when treated with ammonia gas, and became yellow by Hoepfner's reagent and dull green by ferric chloride. From each of these chromatograms this spot was

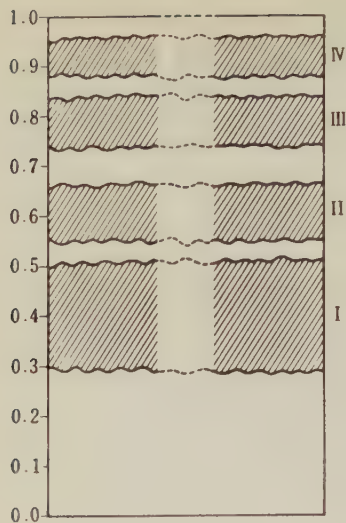


Figure 1. Primary paper chromatogram of *Dahlia* leaf extract.

cut out detecting under ultraviolet light and extracted altogether with warm 90 per cent ethanol. The extract was concentrated and hydrolyzed in a vacuum of a Thunberg tube with N sodium hydroxide solution and after 24 hours standing neutralized with acetic acid to pH 7.2. Of this mixture paper chromatogram was run with the solvent system butanol-acetic acid-water (4 : 1 : 2) and two spots representing caffeic and quinic acid, respectively, were obtained. All these findings may be sufficient for identifying this spot with chlorogenic acid.

From the zones I, II, and IV two other spots of R_f values 0.78—0.83 and 0.71—0.73, respectively, were found above that of chlorogenic acid. The former of them was identified with caffeic acid, and the latter may probably identical with isochlorogenic acid which was found by Burnes, Feldman, and White (3) in coffee beans, although these workers did not state its R_f value. In order to confirm this, an extract of green coffee beans was paper-chromatographed as control, and there were found three spots ($R_f=0.84$, 0.73, and 0.63, respectively) representing caffeic acid, most probably isochlorogenic acid, and chlorogenic acid, the former two of which were identical with the two above mentioned. No other spot which reacted with ferric chloride could be found.

(Procedure B). One thousand grams of fresh *Dahlia* leaves were extracted with 5 l cold ethanol and the filtered extract was concentrated to 100 ml. This was exhausted with ether which left on evaporation white crystals. The crystals proved to be identical with caffeic acid by direct comparison. The aqueous extract was then

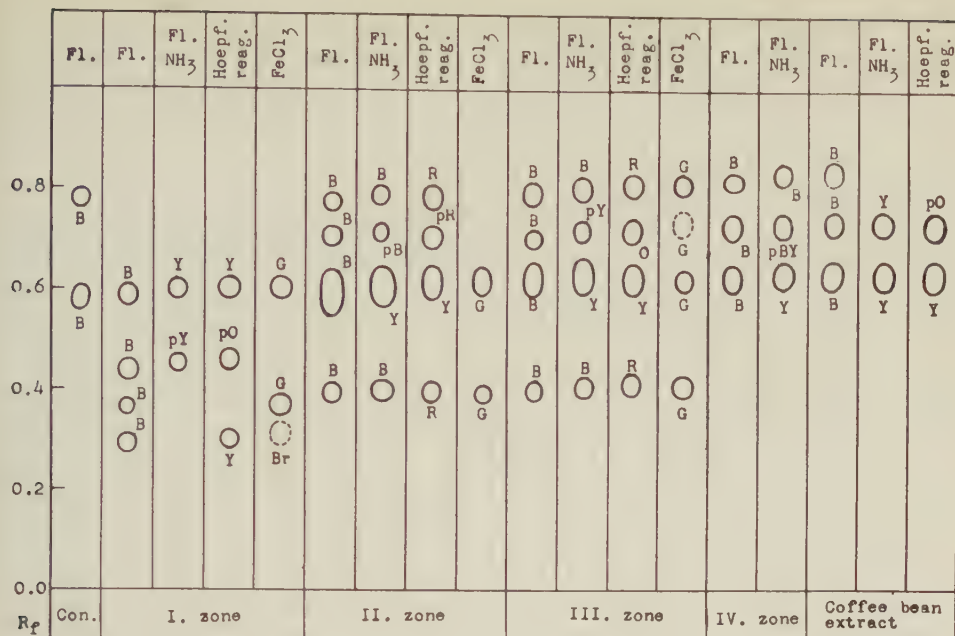


Figure 2. Chromatograms of I, II, III, and IV zones, respectively, as shown in Figure 1. Fl, fluorescence under ultraviolet light; Fl. NH₃, fluorescence under ultraviolet light after treating with ammonia gas; Hoepf. reag., Hoepfner's reagent; FeCl₃, reaction with ferric chloride; B, blue; Br, brown; Y, yellow; pY, pale yellow; pO, pale orange; G, green; pB, pale blue; R, red; pR, pale red; pBY, pale bluish yellow.

exhaustively extracted with ethyl acetate. This extract gave a green color with ferric chloride and yellow precipitate with lead acetate. Despite all attempts, we failed in bringing chlorogenic acid into crystalline state. Hence it was subjected to paper-chromatographic separation by the above mentioned procedure, and the same fractions were obtained.

The aqueous layer, after extracting with ethyl acetate, was mixed with 300 ml. methanol and the insoluble was filtered off. The methanolic solution was concentrated under diminished pressure to about 50 ml. and gave on standing colorless crystals of mineral salts (about 5 g.).

After filtering these crystals, another colorless crystalline substance was obtained in a small quantity and proved to be identical with citric acid after purification by recrystallisation from water.

A third crystalline substance then separated from the mother liquor of citric acid. This was recrystallized from water to give white minute needles of m.p. above 280° (about 0.2 g.) which were identical with tyrosine as compared with authentic specimen on paper-chromatogram.



Figure 3. Chromatograms of the extracts each of untreated leaves, those treated at 100° for 5 minutes, and those treated at 60° for 5 minutes.

Contr., Control; I, IV, Extract of untreated leaves; II, Extract of leaves treated at 100° for 5 minutes; III. Extract of leaves, which became black after heating at 60° for 5 minutes;

M, methionine; T, tyrosine; Al, alanine; G, glycine; gl, glutamic acid; As, aspartic acid; Lac, lactic acid; Malo, malonic acid; Oxa, oxalic acid; Suc, succinic acid; mal, malic acid; Cit, citric acid; Tar, tartaric acid; Caf, caffeic acid; Chl, chlorogenic acid; Cit, citric acid.

2. Behavior of chlorogenic acid and caffeic acid in the leaves while browning

In an attempt to see how chlorogenic as well as caffeic acid behaves in leaves during the course of browning, the following experiments were carried out. Two lots of each 10 g. leaves were heated in a thermostat at 100° and 60°, respectively, for 5 minutes. The former leaves remained green owing to the destruction of oxidizing enzyme, and the latter readily became black owing to the oxidation of these acids. Both of them were then minced in a blender and the juice was paper-chromatographed using butanol-acetic acid-water (4:1:2) by descending method. Chlorogenic and caffeic acid utterly disappeared, while in green leaves they are still present. It is very interesting to notice here, that in the leaves, which turned brown, glutamic acid almost disappeared, and on the other hand, alanine considerably increased (Figure 3).

In order to see whether or not glutamic acid give any favorable effect to the browning reaction of chlorogenic acid, the following preliminary experiment was made.

One ml. of 0.1 per cent solution of chlorogenic acid, 1 ml. of 2 per cent solution of polyphenoloxidase prepared from the leaves of Dahlia and tobacco, and 1 ml. of Melvaine's buffer solution of pH 6.8 were left for 1 hour with

or without 1 ml. of a 0.5 per cent solution of glutamic acid in $N/5$ Na_2HPO_4 . The brown color was roughly twice strong in the experiment with glutamic acid as in that without it.

3. Isolation of chlorogenic acid from coffee beans

For the isolation of chlorogenic acid from coffee beans, Moores, McDermott, and Wood (13) applied isopropanol as extracting solvent. We could not, however, find necessity of using this solvent, but found that ethanol was quite fit. The procedure is as follows.

Two hundred and fifty g. of Javanese coffee beans (not roasted) were pulverized and extracted with ether. The ethereal extract left after evaporation white crystals (8.5 f.), which melted after recrystallisation from water at 223° . This melting point well coincided with that of coffeine and the mixed melting point test proved the identity of this crystalline substance with coffeine. The coffee bean powder, after extraction of coffeine and oily substance, was repeatedly extracted with boiling ethanol until ferric chloride gave no more reaction with the extract. The liquid was condensed by distilling under diminished pressure, when white crystals precipitated. These crystals melted at 240° and gave positive ferric chloride reaction, and proved to be the complex of potassium chlorogenate and coffeine (13) as described below.

This complex product (1.8 g.) was added to a solution of 4 ml. of a tartaric acid solution (24 g. acid in 235 ml. water) and heated for 10 min. and then put in a refrigerator for a short while, when potassium bitartrate separated. After filtering by suction, the liquid was exhaustively shaken with chloroform to remove coffeine. Coffeine thus isolated melted at 228° ; yield about 0.2 g. From the aqueous solution there precipitated chlorogenic acid after several days standing in a refrigerator. Yield about 0.65 g. M.p. 204° . All the reactions and R_f values of this product were the same as those of chlorogenic acid described in literature.

4. Preparation of caffeic acid

Four g. of chlorogenic acid were heated in a mixture of 8 ml. 10 per cent aqueous caustic potash and 10 ml. water 30 min. at 30° followed by neutralisation with 2 per cent sulfuric acid. This solution was heated with charcoal and filtered. From the filtrate were obtained 4 g. of chlorogenic acid of m.p. 180° .

5. Oxidizability of some phenolic substances with crude polyphenoloxidase with or without ascorbic acid

Five g. of dry leaf powder of Dahlia, which had been prepared by mincing the fresh leaves in a blender with acetone or ethanol followed by drying in a desiccator, was extracted with 50 ml. water for 50 hours at 28° with the addition of thymol as disinfectant, and the filtered solution was used as the oxidase solution. As in the case of oxidation of dopa by means of leaf

Table 1. *Oxidizability of various substrates by polyphenoloxidase of Dahlia leaves.*

Chlorogenic acid	+	(Brown)
» » +ascorbic acid	—	
Pyrogallol	+	(Brown)
» +ascorbic acid	—	
Dopa	+	(Black)
» +ascorbic acid	—	
Catechol	+	(Brown)
» +ascorbic acid	—	
Dahlia leaf extract	+	(Brown)
» » » +ascorbic acid	—	
Caffeic acid	+	(Brown)
» » +ascorbic acid	—	
Rutin	—	
» +ascorbic acid	—	
Tyrosine	—	
» +ascorbic acid	—	

Legend: Oxidase solution, 1 ml. Substrate, 0.5 per cent, 1 ml. Ascorbic acid, 1 ml. (=0.560 mg.) McIlvaine's buffer solution, pH 6.8, 1 ml. 14 hours, at room temperature.

polyphenoloxidase (2), ascorbic acid inhibited the oxidation of the substrates. This phenomenon was also observed recently by Weurman and Swain (21). The results are shown in Table 7.

It is noteworthy that neither rutin, although it has 3,4-dihydroxyphenyl group, nor tyrosine was oxidized. In this connection this oxidase may be a polyphenoloxidase similar to what we reported in the case of dopa of *Stizolobium* (2).

6. Presence of chlorogenic acid in several plants

There are many species of plants that show strong or more or less strong death-ring formation. Of these we investigated eight species by means of paper-chromatographic method as to the presence of chlorogenic acid with positive results in every case (Figures 4, 5). Those were *Aralia cordata*, *Aucuba japonica*, *Clematis paniculata*, *Cryptotaenia japonica*, *Ilex latifolia*, *Inula salicina*, *Spiraea japonica* var. *ovatifolia*, and *Viburnum Sargentii*, and about 10 g. each of their leaves were extracted with boiling ethanol (50 ml.) three times, the combined extract concentrated by distillation, and the concentrate was chromatographed on filter paper. Sometimes when the spots separated difficultly, this concentrate was several times shaken with ethyl acetate, the combined ethyl acetate layer distilled off, and the residue paper-chromatographed after dissolving in a few ml. water. Besides chlorogenic acid, caffeic acid was also found in *Clematis paniculata* and *Viburnum Sargentii*.

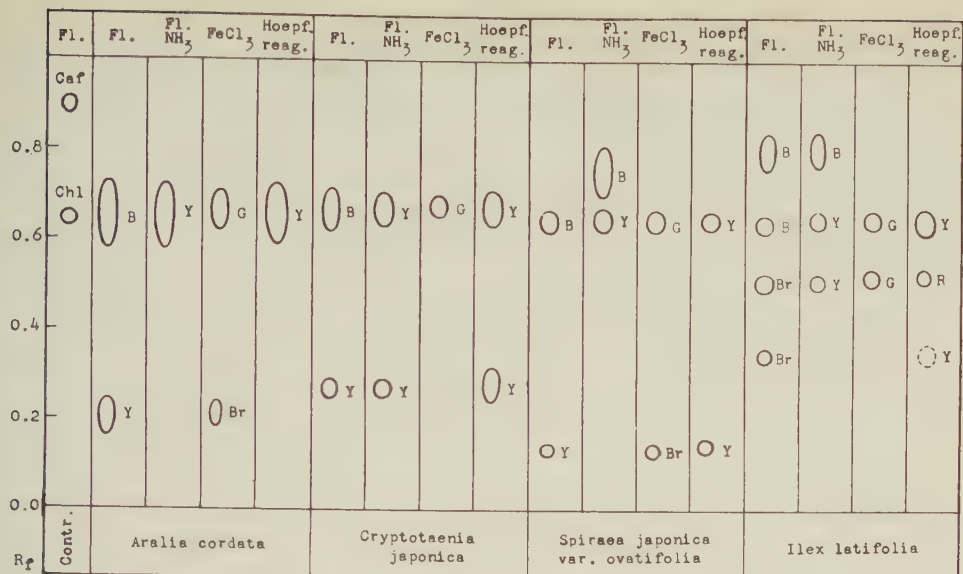


Figure 4. Chromatograms of the leaf extracts of *Aralia cordata*, *Cryptotaenia japonica*, *Spiraea japonica*, var. *ovatifolia*, and *Ilex latifolia*, respectively. — Abbreviations as in Figures 2 and 3.

In the case of *Inula salicina* a distinct spot, the R_f value of which, however, completely coincided with that of caffeic acid, was found, but its color reaction is quite different from those of the latter.

Among these examples *Aucuba japonica* is quite interesting in that it contains both aucubin and chlorogenic acid. Aucubin itself is very stable against polyphenoloxidase and splits into one mole each of aucubigenin and glucose when hydrolyzed by mineral acids or β -glucosidase. The aglycone aucubigenin is on the contrary very unstable in the air and changes in the acid solution, in which it was formed, on spontaneous oxidation into a black substance of unknown structure. The blackening reaction of the leaves of this plant is accordingly ascribed to the hydrolysis of aucubin and oxidation of aucubigenin formed (8). As there has been found chlorogenic acid, it must be examined whether or not this substance also plays a role in the blackening of *Aucuba* leaves together with aucubin.

Fresh leaves of *Aucuba* were minced with 90 per cent ethanol and the tissue powder repeatedly extracted with cold ethanol until all of aucubin and chlorogenic acid were removed. For the detection of aucubin, ethanolic extract was each time hydrolyzed with hot hydrochloric acid and examined if the solution became black, and chlorogenic acid was tested with ferric chloride solution.

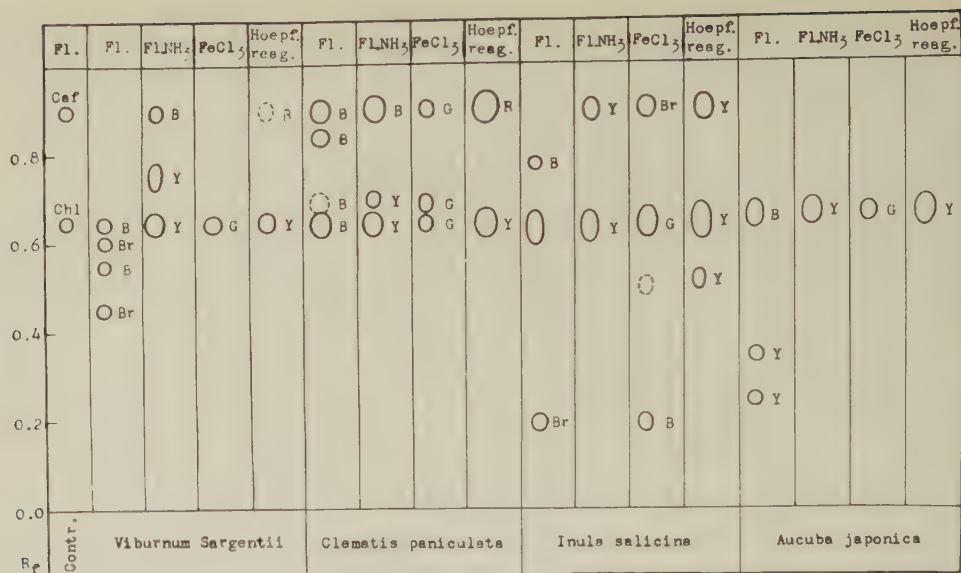


Figure 5. Chromatograms of the leaf extracts of *Viburnum Sargentii*, *Clematis paniculata*, *Inula Salicina*, and *Aucuba japonica*. Abbreviations as in Figures 2 and 3.

When chlorogenic acid was treated with aqueous suspension of this *Aucuba* leaf powder thus prepared, no color change occurred, showing the absence of any polyphenoloxidase. Thus it was established that chlorogenic acid does not take part in the blackening process of *Aucuba* leaves.

Discussion

Chlorogenic acid was discovered by Robiquet (18) in 1837 in unroasted coffee beans as an acid, which gave a green coloration with ferric chloride. Thus acid was then isolated by Rochleder (19), who named it »Kaffeegerbsäure«, but Payen (15), who studied it more elaborately, gave it the name »acide chlorogénique« on behalf of its difference from tannin and the green color it gave on the addition of ferric chloride. Quite indifferent from these authors, Ludwig and Kromayer (12) isolated in 1859 from sunflower seeds an acidic substance in amorphous state and gave it the name »Helianthsäure«. This acid was proved to be identical with chlorogenic acid by the work of Gortler (7). Gortler (6) a little earlier (1909) published a paper on the wider occurrence of chlorogenic acid in higher plants by color test with ferric chloride. According to his description 227 species of 71 families from Cyathe-

aceae to Compositae were examined and 92 species proved to contain chlorogenic acid.

In 1910 Charaux (4) also investigated the distribution of the acid and found it in 33 out of 42 species belonging to 11 families. Politis (16) developed a new method for identification of chlorogenic acid, which consisted in immersing plant tissues in 20 per cent ammonia for 10—20 hours and adding thereupon a drop of sulfuric acid. When chlorogenic acid is present, the color will be reddish purple, and in the material he used, that is, three species of *Chrysanthemum*, *Ch. frutescens*, *Ch. leucanthemum*, and *Ch. japonicum*, the ligulate flower petals were demonstrated to contain chlorogenic acid, while those of *Rosa* sp. (white flowered) and *Spiraea* sp. did not contain it. Politis (17) further investigated the distribution of chlorogenic acid in various organs of 42 solanaceous plants.

Recently, chlorogenic acid has drawn not a little attention in view of food preservation, particularly that of fruits, because this acid might be the cause of their browning. From this standpoint Hulme (11) succeeded in isolating the acid in pure state from apples.

In this connection, Weurman and Swain (21) have also made probable that chlorogenic acid might be one of the browning agents in apples and pears. In fact, Bradfield, Flood, and Williams (2) isolated this acid from ripe and unripe apples, pears, and leaves as well as shoots of pear trees. In the leaves and shoots of pear tree, it was accompanied by isochlorogenic acid, which had been isolated from coffee beans by Burnes, Feldman, and White (3). Corse (5) recently isolated another isomer of chlorogenic acid, neochlorogenic acid, from peaches. Isochlorogenic acid and neochlorogenic acid which are believed to differ from chlorogenic acid in that the position of the ester linkage in the former two is different from each other and from the former. The *o*-dihydroxyphenyl group of these three isomeric acids may be responsible for the dull green color produced by ferric chloride and for the oxidation by polyphenoloxidase.

The work of Gorter, Charaux, and Politis were solely based on the color reactions, which, although to some extent reliable, involve some uncertainty in identifying chlorogenic acid. For this reason the need for reinvestigation on the distribution of chlorogenic acid in plant kingdom by means of paper-chromatography is keenly felt.

It is not free of danger if we postulate chlorogenic acid, and possibly the two isomers and in some cases also caffeic acid as well, as the sole browning agents in plant tissues, because some plants contain aucubin (8), and others contain dopa (9) as browning agents. Besides *Dahlia* we have eventually confirmed the presence of chlorogenic acid in the leaves of *Aralia cordata*, *Aucuba*

japonica, *Clematis paniculata*, *Cryptotaenia japonica* var. *ovatifolia*, *Ilex latifolia*, *Inula salicina*, *Spiraea japonica*, and *Viburnum Sargentii* by paper-chromatographic method, and among them the three species, *Clematis*, *Inula*, and *Viburnum* contain also caffeic acid.

Recently, Abumiya and Kobayashi (1) pointed out that the brown spots which are usually formed on the leaf blades of rice plant infected by a parasitic fungus, *Piricularia oryzae*, are caused by the oxidation of chlorogenic acid. A more interesting and noteworthy observation was made by Tamari and Kaji (20), who established in rice plant the presence of an antagonistic activity of chlorogenic acid against the growth-inhibiting action of piricularin, one of the toxic principles exuded by *Piricularia oryzae*.

There has been some discussion about the physiological significance of chlorogenic acid, for example, its possible participation in plant respiration, which dates back to perhaps Oparin (14), but we confined ourselves in this report to its distribution and role as the browning agent in leaves.

Summary

1. Leaves of *Dahlia variabilis*, *Aralia cordata*, *Clematis paniculata*, *Cryptotaenia japonica*, *Ilex latifolia*, *Inula salicina*, *Spiraea japonica* var. *ovatifolia*, and *Viburnum Sargentii* contain among others chlorogenic acid as the agent, which gives rise to brown substance when subjected to oxidation by polyphenoloxidase. *Dahlia*, *Clematis*, and *Viburnum* contain besides chlorogenic acid caffeic acid which is also oxidizable by this enzyme. The presence of these acids were detected by paper-chromatography.

2. Leaves of *Aucuba japonica* do not contain oxidase, although they contain chlorogenic acid.

3. Oxidation of chlorogenic acid by oxidase is inhibited to some extent by ascorbic acid.

4. In the leaves which had been allowed to stand at about 60° for oxidation for 5 minutes and become dark brown, chlorogenic acid and caffeic acid completely disappeared.

5. From leaves of *Dahlia* citric acid and tyrosine were isolated.

6. In leaves which had changed into dark brown and in which chlorogenic acid had completely disappeared, glutamic acid almost completely disappeared and the quantity of alanine, which had been present only in a small amount, considerably increased.

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References

1. Abumiya, D. & Kobayashi, T.: Read before the General Meeting of the Phytopathological Society of Japan. — 1954.
2. Bradfield, A. E., Flood, A. E., Hulme, A. C. & Williams, A. H.: Chlorogenic acid in fruit trees. — *Nature* 170: 168. 1952.
3. Burnes, H. M., Feldman, J. B. & White, W. V.: Isochlorogenic acid. Isolation from coffee and structure studies. — *J. Amer. Chem. Soc.* 72: 4178. 1950.
4. Charaux, C.: Sur l'acide chlorogénique. Fréquence et recherche de cet acide dans les végétaux. Extraction de l'acide caffeique de quelques plantes. — *J. Pharm. Chim.* 2: 191. 1910.
5. Corse, J.: A new isomer of chlorogenic acid from peaches. — *Nature* 172: 771. 1953.
6. Gorter, K.: Ueber die Verbreitung der Chlorogensäure in der Natur. — *Arch. Pharmaz.* 247: 184. 1909.
7. — Zur Identität der Helianthsäure mit der Chlorogensäure. — *Arch. Pharmaz.* 247: 436. 1909.
8. Hattori, S. & Kurihara, M.: Studies on death-ring. I. On an enzyme hydrolyzing aucubin in the leaves of *Aucuba japonica*. — *Misc. Rep. Res. Inst. f. Nat. Resources*. Nos. 17—18. 1950.
9. — & Shiroya, M.: Studies on the browning and blackening of plant tissues. II. On the interaction of dopa and a specific oxidase in the leaves of *Stizolobium Hassjoo*. — *Physiol. Plantarum* 8: 63. 1955.
10. Hoepfner, W.: Zwei neue Reaktionen für Kaffeesäure und Chlorogensäure. — *Chem. Ztg.* 56: 991. 1932.
11. Hulme, A. C.: The isolation of chlorogenic acid from the apple fruit. — *Biochem. J.* 53: 337. 1953.
12. Ludwig, H. & Kromayer, O.: Untersuchungen der Samen von *Helianthus annuus*. — *Arch. Pharmaz.* 99: 1. 1859.
13. Moores, R. G., McDermott, D. L. & Wood T. R.: Determination of chlorogenic acid in coffee. — *Anal. Chem.* 20: 620 1948.
14. Oparin, A.: Das grüne Atmungspigment und seine Bedeutung bei der Oxydation der Eiweisskörper in den keimenden Samen des *Helianthus annuus*. — *Biochem. Ztschr.* 124: 90. 1921.
15. Payen: Mémoire sur le café (Deuxième partie). — *Compt. rend. Acad. Sci. Paris*. 23: 8. 1847.
16. Politis, J.: Sur une nouvelle methode concernant la localisation microchimique de l'acide chlorogénique et des tanins dans les plantes. — *Compt. rend. Acad. Sci. Paris* 225: 954. 1947.
17. — Sur la distribution de l'acide chlorogénique dans la famille des solanacées et dans les organes de ces plantes. — *Compt. rend. Acad. Sci. Paris* 226: 692. 1948.
18. Robiquet, P. J. & Boutron, A. F.: Ueber den Kaffee. — *Lieb. Ann. Chem.* 23: 93. 1837.
19. Rochleder, F.: Untersuchung der Kaffeebohnen. — *Ann. Chem.* 50: 224. 1884.
20. Tamari, K., & Kaji, J.: Biochemical studies on the toxins of rice blast fungus, *Piricularia oryzae* Cavara (in Japanese). — *Biol. Science (Japan)*, 6: 73. 1954.
21. Weurman, C., & Swain, T.: Chlorogenic acid and enzymic browning of apples and pears. — *Nature* 172: 678. 1953.

A Development Stage in *Fagus Silvatica* Characterized by Abundant Flowering

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Artificial pollination plays an important part in tree breeding. Grafts of selected trees of most tree species have proved capable of flowering satisfactorily to carry out artificial pollination experiments. Grafts of *Fagus silvatica* however rarely produce flowers and during the period 1948—54 some experiments of a prospective formation of flower buds were carried out at the Arboretum of Hørsholm. The experiment were made on the classical lines of horticulture viz. manuring, girdling, ringing and root pruning, but so far with negative results. The existence of juvenile and other development stages in beech were however not taken into account in these experiments.

Observations of occasional flowering in grafts of *Fagus* however indicated the importance of choosing the correct grafting material. Only 6 grafted clones out of about 90 produced female flowers even when the exceptionally good seed year 1954 is included. In 1954 these 90 clones comprised more than 3000 grafts propagated before 1953. The majority of grafts even within these 6 clones carried no flowers. In reviewing these observations it was noted that some scionwood from the 6 parent trees had in many cases been collected in flowering parts of the crowns. In order to get more exact information the following experiments were carried out.

Experiments

In the early spring of 1952 scions with many flower buds were collected from V. 1272, a very old beech which had flowered every year since 1948.

Table 1. *Flowering of the clone V. 1272 in 1954.* When grafted on horizontal branches facing south west of an old isolated tree: abundant flowering grafted on young trees: no flowering.

Rootstock	Number of grafts		Total number of female flowers 1954	Male flowers 1954	Vigour of the grafts
	grafted 1952	alive 1954			
50 year old tree	50	22	452	Abundance	In general fairly good growth
Young trees ...	20	7	None	None	In general extremely vigorous growth

In May 50 scions were grafted on horizontal branches facing south west of a 50 year old tree which had flowered in 1948 and which grew in a sunny glade. 20 scions were grafted on 3 vigorous 10 year old grafts of one young clone of *Fagus orientalis*. These 3 plants also grew in isolation but were somewhat less exposed to direct sunlight. When grafted these trees were cut back more severely than the stock branches of the old tree, and 5 of the 7 grafts, which were still alive in 1954, grew far more vigorously than any of the 22 live grafts on the old stock. In the 2 remaining grafts the graft-union was not satisfactorily established.

Table 1 shows that there was abundant flowering in 1954 in the grafts on the old stock but no flowering occurred on the young ones. Female flowers were always produced in conjunction with male flowers.

All grafts flowered on the old stock, but it was observed that the most vigorous grafts produced relatively few female flowers.

In 1952, 4 other clones were grafted on the same old isolated tree, some of them on branches very exposed to sunlight but, as shown in table 2 no flowering grafts were observed in 1954.

Table 2. *Beech clones grafted on an old isolated tree in May 1952.* Only material from flowering branches of the very old tree V. 1272 flowered in 1954.

Clone number	Number of live grafts 1954	Number of female flowers		Remarks
			1954	
V. 1272	22		452	Scions with many flower buds
V. 266	3		None	Scions without flower buds
V. 263	28		None	" " " "
V. 884	6		None	" " " "
V. 440 A	7		None	Scions in the leaf-retaining stage, without flower buds
V. 440 B	12		None	Scions in the leaf-shedding stage, without flower buds

Discussion

Whilst the grafts of V. 1272 on young stocks were transformed into a vegetative state, the grafts on the old tree remained in their flowering state. This proves the importance of exogenous influences. The most important factor seems to be the choice of rootstock material, but it must be admitted that environmental factors, such as exposure to sunlight, may play an important part. It is a common and reliable observation that beech crowns exposed to sunlight flower more frequently and more richly than beech crowns in shade. But as the old rootstock was unable to force the 4 other clones to flower, it was obvious *that the abundant flowering of clone V. 1272 must primarily be due to a strong capacity for flowering in the scions proper.*

It is common experience that old isolated beeches may flower every year although a high fruit production is found only in certain years which however occur more often than normal mast years in beech. V. 1272 and another old tree in the Jægersborg State Forest District thus flowered each year during the period 1948—54 with heavy masts in 1948, 1950, 1952 and 1954. The author believes that *old beeches will pass into a stage characterized by abundant and frequent flowering.* It should be borne in mind however that characteristics of flowering, i.e. earliness and abundance, are known to be hereditary in birch (Johnsson, 1949) and may be hereditary in many tree species. The experiments referred to in this paper prove the possibility of keeping branches at this stage by ensuring optimum external factors for flower production. Unfavourable conditions for the formation of flower buds on the other hand immediately modified the scions into vegetative shoots. This stage of development thus proved far less well established than the leaf-retaining juvenile stage (Schaffalitzky de Muckadell, 1954).

Although the material is not sufficiently large to prove the contrast between vigorous vegetative growth and the production of flowers, the tendency is obvious. This tendency has often been mentioned in literature and J. D. Matthews (unpublished) has kindly informed me of similar experiences with beech grafts in Great Britain. Grafts showing a very poor vegetative growth, however, generally seem too weak to produce flowers.

The few cases of flowering in grafts on young rootstocks previously mentioned show that the capacity to flower may rarely be retained in this way. In the above experiments some flower buds might have been produced in the grafts on young stocks if the latter had been less vigorous than the *Fagus orientalis* used. The maintenance of the flowering ability in a scion thus appears to be the result of a reciprocal interaction between scion and stock.

Even when the scion originates from flowering parts of a beech crown it generally appears difficult to establish a reciprocal interaction favourable for the formation of flower buds.

Summary

Grafts of *Fagus silvatica* were kept in a flowering development stage by using horizontal branches facing south west of a 50 year old isolated tree, as rootstock. The flowering stage proved to be poorly established in control experiments with grafts on young stocks.

References

- Johnsson, H.: Hereditary precocious flowering in *Betula verrucosa* and *Betula pubescens*. — *Hereditas* 35: 112. 1949.
- Schaffalitzky de Muckadell, M.: Juvenile stages in woody plants. — *Physiol. Plant.* 7: 782. 1954.

The Connection between the Respiratory Gradient and the Growth Rate in Wheat Roots

By

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Introduction

The extension growth is, in addition to water uptake, accumulation of osmotically active material and wall growth, also connected with intensive formation of new cytoplasm (Brown and Broadbent, 1950). The cells in the extension zone are also characterized by a high metabolic rate. Their respiration measured as oxygen uptake on a protein-nitrogen or total nitrogen basis, often exceeds the respiration of the embryonic cells to a very essential degree as is shown in some investigations on root material. Kopp (1948), who recalculated on a protein basis the respiration values in different sections of wheat roots determined by Wanner (1944), found that the oxygen uptake in the extension zone was nearly three times as great as that of the meristematic zone. Wanner (1950) has obtained similar results for roots of *Allium Cepa* and Baldovinos (1950, 1953) for roots of *Zea mays*. Goddard and Meeuse (1950) report that the respiration on a nitrogen basis in roots of *Zea* is low in the meristematic zone, rises strongly and reaches a maximum in the extension zone and sinks to a constant level after the cessation of the growth. Brown and Broadbent (1950), on the other hand, did not find so marked differences in the respiration rates for different growth zones of *Pisum* roots. That there is a considerable increase in the respiratory intensity on a nitrogen basis is, however, confirmed for wheat roots by results presented in this paper.

Thus the transition of a cell from the embryonic state to the extension state normally involves a more intense energy turnover. The cause of this often very

striking increase in the respiration has not been subjected to any greater attention. The problem of the connection between growth and energy turnover has instead been coupled together with the question of the mechanism of action of the growth substances. Avery (1951) is thus of the opinion that the problem of the relations of the auxins to the respiration may be solved in the best way by studying material that, as a result of auxin treatment, will give both changes in growth and in respiration. Later reviews and discussions of the effect of auxins on the metabolism are given by Bonner and Bandurski (1952) and Gordon (1953). In spite of extensive investigations it has not been possible to establish that the auxins have any direct effect on the oxidation processes in the cell. In any case there is strong evidence that the increase in respiratory intensity, which is taking place in root cells passing into the extension state, hardly can be caused by such an effect. Thus Audus and Garrard (1953), who investigated the effect of β -indolylacetic acid (IAA) on the respiration of excised root sections of pea, found no direct effect of IAA, either in growth-stimulatory or growth-inhibitory concentrations. On the other hand, they established that the respiration during the growth was strongly correlated with the length of the sections. Probably there is no corresponding increase in protein (cf. Robinson and Brown 1954). The increase in the respiratory rate without a corresponding increase in protein must evidently be connected in some way or other, with the changes in the cells during the extension growth. Probably it is the same case with the respiratory increase, which has been found in stem tissue, connected with an auxin-induced increase of the extension growth (cf. Audus and Garrard 1953). This stimulation is for the most part rather slight compared with the respiratory increase obtained by Kopp (1948) and Wanner (1950) for roots at transition from growth by cell multiplication to extension growth. Bonner (1949) thus reports that the increase in respiration, induced by IAA in growth-stimulatory concentrations, in excised *Avena coleoptiles* amounts to 10—35 per cent, whereas Christiansen and Thimann (1950) state the corresponding increase in etiolated pea stem sections to 15—25 per cent. However, it should be noted that the comparison in these cases is made with cells that probably already have begun extension growth but are elongating more slowly (cf. Brown, Reith, and Robinson 1952). Another important difference, which must be considered in comparing tissues from stems and roots, is that the extending zone in the former is very prolonged and for this reason the growth rate of the individual cells is low compared with the case in roots, where the extension growth is concentrated to a relatively short zone.

The investigation presented in this paper is an attempt to elucidate the question of how the increase in the respiratory rate connected with the extension growth is influenced by the rate of the root elongation. The method used has been to determine the respiratory gradient in roots grown with different growth rates. As means of regulating the magnitude of the elongation the following have been employed: on the one hand IAA in growth-inhibitory concentrations and on the other hand α -3-indole-isobutyric acid (HBA) that produces a strong increase of the growth (Burström 1951 a). However, the question is to what extent the growth substances used control the growth without directly influencing the metabolic processes. The answer to this question is important for the interpretation of the results. It is closely connected

with the problem of the mechanism of action of auxins and will be brought up for further discussion in conjunction with an analysis of the relations between cell length, fresh weight, dry weight and organic nitrogen during the growth.

Methods

The experimental material consisted of roots of wheat seedlings (Weibulls orig. »Eroica»), which were grown in sterile nutrient solution. The seeds were pretreated by soaking in 0.1 per cent sublimate-formaldehyde solution and sterilized by calcium hypochlorite solution according to a method earlier described by Almestrand (1949). The solution of hypochlorite used, however, was much more diluted. It was made by dissolving 40 gm calcium hypochlorite in one litre of water and filtering the solution after which the filtrate was diluted ten times.

This diluted solution had, as a matter of fact, the same sterilizing effect as the undiluted solution without having the variable effect of the latter on the sterilization and the fertility of the seeds. The reason for this is that both the sterilization and the poisonous effect of the hypochlorite solution depends on the pH of the solution. This is due to the fact that the effective constituent is free HClO. The pH value of the solution is high from the beginning (about 12) but is lowered by the carbon dioxide evolved from the seeds. As a consequence of this the proportion of HClO is strongly increased during the soaking. If the pH is maintained at 12 the seeds may be kept in an undiluted solution for more than 40 hours without injury. At pH 8 the same solution will kill them in less time than one hour. Consequently there are many factors difficult to control which influence the effect of the hypochlorite solution, for example, the amount of solution for a given amount of seeds and the carbon dioxide output of these, which will vary with the initial moisture content. Small variations in these factors may give large differences in the effect of the hypochlorite solution. If a sufficiently diluted solution is used the pH value will fall rather rapidly to near the neutral point where it will remain approximately constant. In this case the sterilization of the material will be relatively uniform in different samples without injuries to the grains.

The grains were germinated in sterile Petri dishes for two days in the dark at 22° C. Then the seedlings were transferred to sterile nutrient solutions in wide-necked Erlenmeyer flasks. These were fitted with a cotton plug through which ran a capillary tube. The lower part of this served as a holder for the seedlings by means of repeated curvatures. The capillary tube was also used for aerating the nutrient solution, the air passing a cotton filter in another tube. In the transferring procedure the cotton plug with the holder was taken out of the flask. This method is a modification of a similar method used by Almestrand. In each flask 10 seedlings were cultured in 500 ml nutrient solution. The cultivation was carried out in the dark at 22° C for two days. The composition of the nutrient solution in mmol per litre was: KNO_3 0.2, KH_2PO_4 0.3, $\text{Ca}(\text{NO}_3)_2$ 0.4, MgSO_4 0.2, MnCl_2 0.01, H_3BO_3 0.001, and $\text{Fe}_2(\text{SO}_4)_3$ 0.01. The pH of the solution was adjusted with KOH and H_2SO_4 to 5.0—5.2. The flasks were sterilized with the solutions in an autoclave at 110—115° C for 20 minutes. The growth substances used were dissolved in water containing an equivalent quantity of KOH. This solution was sterilized by filtering through a sterile-filter and introduced into the culturing vessels after these had been auto-

claved. The technique employed for sterile culturing was effective for avoiding bacterial infections in the solutions, but it was not possible to avoid completely fungus infections on the grains. No influence on the respiration of the roots was, however, noticed as a consequence of these.

The oxygen uptake was determined at 25° C in a Warburg apparatus according to the description of Umbreit et al. (1949). The flasks were shaken at a rate of 110 oscillations a minute. Variation of the shaking rate between 95 and 150 oscillations a minute did not cause any differences in the oxygen uptake. This was, furthermore, proportional to the amount of roots in the flask, and consequently there is no reason for suspecting that the oxygen supply may have been a limiting factor for oxygen uptake. The preparation of the roots like the respiratory determinations was performed in normal light. Only the three earliest developed roots were used in the experiments. A suitable number of roots was collected on a moistened glass plate, arranged with the tips at the same level and cut into segments with razors. The lengths of these segments were in order from the tip 2 mm, 2 mm, 3 mm, 3 mm and from the remainder of the root 10 mm. The segments were successively transferred to watch-glasses with nutrient solution or moistened filter paper. The time necessary to cut roots for an experimental series was about an hour. As »cutting time» was taken the point halfway between the beginning and completion of the preparation. The segments from the same position in the root were well mixed before they were distributed between different parallel samples. They were surface-dried on filter paper and their fresh weight was determined by rapid weighing on a torsion balance. Then they were transferred into the Warburg flasks and suspended into 2 ml nutrient solution of the same composition as the growth solution. In the sidearm 0.5 ml 0.25 *M* glucose solution was placed. The respiratory determinations began three hours after the cutting of the roots and the oxygen uptake without substrate was determined for three hours. Then the glucose solution was added, the final glucose concentration being 0.05 *M*, and after half an hour for calibration the oxygen uptake was determined for another two hours. The respiration values given in this paper are the oxygen uptake determined in the presence of glucose. The connection between the respiration in wheat root segments with and without substrate is dealt with in another paper (Karlsson and Eliasson 1955).

After their respiration had been measured the segments were dried in vacuum at room temperature and the dry weight and total nitrogen were determined. For these determinations as a rule material from two parallel samples had to be added together. For determinations of the total nitrogen the micro-Kjeldahl method according to Klein (1931) was employed. The determinations of the oxygen uptake have been repeated six to eight times for each root material. The agreement between the different determinations of the oxygen uptake per unit fresh weight has been very good. The nitrogen determinations have been made in two or three series for each root material.

Results

The root growth

The growth rate in roots is determined by the cell multiplication rate and the size of the cell elongation. The former will be influenced very little by

Table 1. *The length of the extension zone in roots with different growth rates.*

Growth substances	Root growth during two days (cm)	Length of the extension zone (mm)
Control	4.1	3.5
IAA (strong inhibition)	1.4	1.3
IAA (slight inhibition)	2.2	2.2
IIBA	5.5	5.3

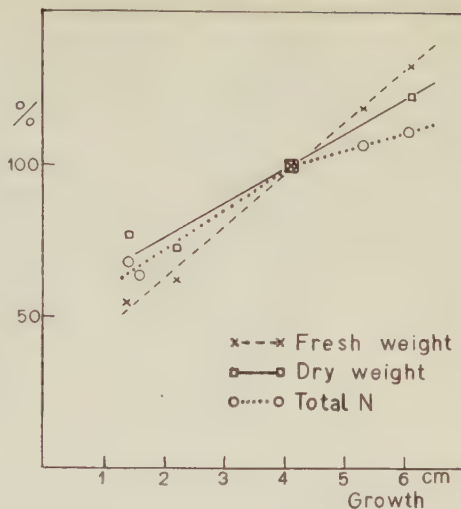
growth regulators in moderate concentrations, while the latter is very sensitive to different factors and may easily be regulated within wide limits with different growth substances (cf. Burström 1951 a, and Hansen 1954). The differences in growth rate obtained by treatment with growth substances are thus caused mainly by differences in cell elongation. However, it is necessary to make a distinction between the growth rate of the roots and that of the cells. The former is determined by the final length of the cells which in its turn depends upon the duration of the cell extension and its rate (cf. Burström 1953). The rate of cell elongation according to Burström (1950) is relatively independent of auxin treatment. The auxins instead determine the length of the growth period for each cell.

In the experiments described in this paper an inhibition of the elongation of the roots by about 50 per cent has been obtained by supplying IAA in the concentration $2-2.5 \times 10^{-8}$ M. On the other hand the growth has been increased by supplying IIBA in the concentration 3×10^{-6} M. The growth increase obtained varied between 30 and 50 per cent. The growth stimulation by more than 100 per cent obtained with the same substance by Burström (1951 a) refers to other growth conditions under which the control grew more slowly.

The elongation of wheat roots is, as a matter of fact, very sensitive to several factors other than supplied growth substances, and it is possible to get an increase in growth similar to the IIBA stimulation by appropriate choice of culture conditions. Rosemark (1954) thus obtained a strong growth stimulation by reducing the nitrogen supply. No further stimulation could be obtained by α -parachlorophenoxy-isobutyric acid, a substance with growth effects similar to those of IIBA. Under the growth conditions used in the experiments described here, an increase in growth of the same magnitude as with IIBA could be obtained by supplying carbon powder to the nutrient solutions. The most probable explanation of this is that the carbon absorbs an inhibitory substance from the solution. Burström (1954) concludes from experiments with growth-stimulatory and growth-inhibitory substances that an unknown factor normally limits the growth of roots.

The length of the growth zones has been determined by measuring cell lengths at different distances from the tip. The points, where the extension zone begins and ends, have been obtained from the growth curve constructed from these values. The length of the meristematic zone is equal in roots with

Figure 1. *The connection between elongation and fresh weight, dry weight and total nitrogen in wheat roots.* Each point on the curves is based on 2—4 experimental series. The values refer to the piece of the root grown during 2 days at 22° C in the dark. Inhibition and stimulation of the growth are due to growth substances supplied to the growth solutions. The abscissa gives the elongation of the roots during 2 days. The values of fresh weight, dry weight, and total nitrogen have been expressed as per cent of the values of control roots.



different growth rate and is together with the calyptra 1.75 mm. The length of the extension zone is, on the other hand, strongly correlated with the growth rate of the roots. Its average length in roots with different growth rate is shown in table 1.

Figure 1 shows how fresh weight, dry weight, and total nitrogen in the piece of the root developed during the two days in the nutrient solution are related to the elongation. For the determinations segments of the same kind as those used in the respiration experiments have been used. The values given in the diagram have been obtained by adding the values for the segments of a root. The tip segment consisting of the meristem has been omitted in order to give a more correct picture of the newly formed tissue. The figure shows that changes in root length are greater than the changes in the other properties. This is especially the case in comparison with dry weight and nitrogen. Similar results have been obtained with the same material by Burström (1951 a). From figure 1 it may be concluded that a fourfold increased elongation corresponds to an increase in the dry weight of only 50 per cent. Similar conditions hold true for total nitrogen. According to Burström (1951 a), an increase in cell lengths of 100 per cent corresponds to an increase in protein nitrogen of only 8—10 per cent. The fresh weight and hence the volume do not increase in proportion to the length. This may be due to a diminishing of the cross-sectional area of the cells at the same time as the cell length increases. The results indicate that the elongation of the cells is primarily regulated by the growth substances, while the changes of the other properties may be presumed to be a consequence of the change of the elongation.

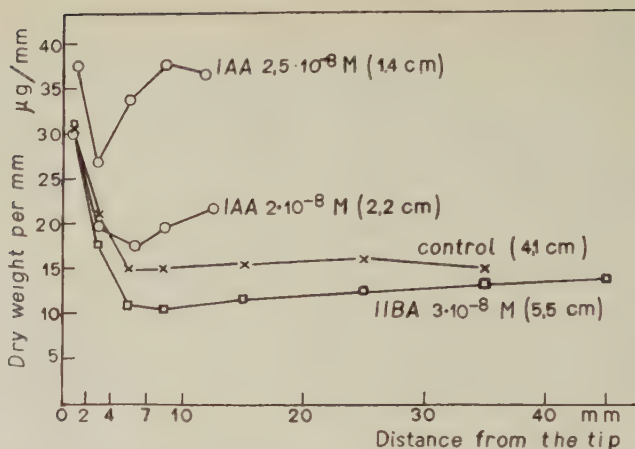


Figure 2. Dry weight per mm of wheat roots. The roots grown for 2 days in dark at 22° C in solutions with growth substances causing different degrees of growth inhibition or stimulation. The figures in parentheses show the growth in length during the culture period. — Mean values for three to five samples with 50—100 root segments in each.

Figure 2 shows how the dry matter in roots with different growth rate is distributed along the roots. Dry weight per unit length is according to this inversely proportional to the elongation. The same holds true for total nitrogen. Consequently the inhibition caused by IAA can hardly be due to a deficiency of growth materials.

Respiration in different parts of the roots

Figure 3 shows how the oxygen uptake varies with the distance from the tip, when calculated per unit length of the root. With this as a reference unit the respiration decreases to less than a half during the extension growth. Then there is a slower decline as the roots become older. The investigations of the respiration in different zones of roots earlier reported have in most cases given the same results. See, for example, Machlis (1944), Wanner (1944), and Brouwer (1954). The differences between roots of different growth rates are for the main part due to differences in the thickness of the roots (cf. figure 2). The high respiration of the IAA-inhibited roots is probably also connected with the intense root hair growth in these roots (cf. Hansen 1954).

The decrease in the respiratory rate during the extension growth may be due to the »dilution» of the living protoplasm, which is a consequence of the water absorption in the extension zone. This water uptake manifests itself in a decrease in the dry matter content in normal roots from 20 per cent in

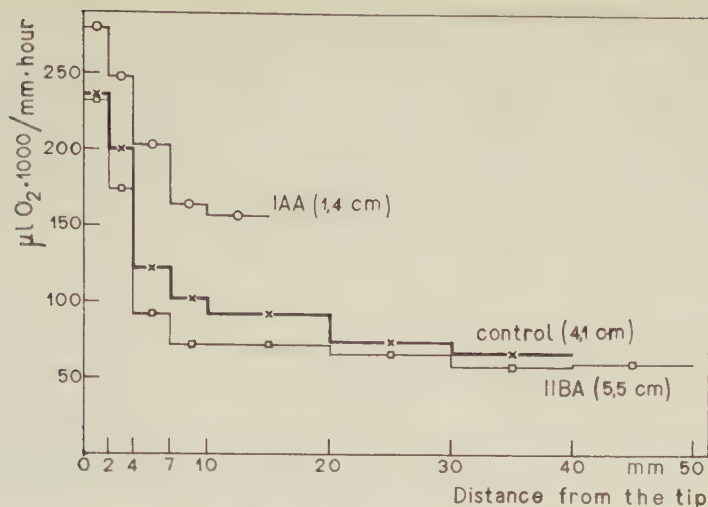


Figure 3. Oxygen uptake per mm root in different parts of wheat roots. The roots grown for 2 days in nutrient solutions with growth substances added. The figures in parentheses show the growth in length during the culture period. The respiration measurements were carried out with the roots in nutrient solution with 0.05 M glucose 6 $\frac{1}{2}$ —8 $\frac{1}{2}$ hours after cutting of the roots.

the meristem to 7 per cent in mature root tissue during the cell elongation. Besides there is also a change in the relation between nitrogen-free and nitrogen-containing substances. While the dry weight per mm root is reduced to one half during cell elongation (figure 2), the total nitrogen is reduced to one fourth. For these reasons it is clear that a comparison of the respiration in different zones of the root will not show the real differences in metabolic activity, if the values are expressed on a length, fresh weight or dry weight basis. As a more appropriate unit protein nitrogen has often been used. While the relation between soluble nitrogen and protein nitrogen according to Kopp (1948) is relatively constant in different parts of wheat roots, total nitrogen may be considered as approximately proportional to the protein content.

Figure 4 shows the oxygen uptake per mg total nitrogen in the same kind of roots as in figure 3. The oxygen uptake shows on this basis a considerable increase in the extension zone which is nearly as great as that earlier reported by Kopp (1948). This increase is followed by a decrease setting in later on after the completion of the cell extension. Probably the oxygen uptake in purely meristematic tissue is somewhat lower than in the tip segment used here, as this zone also includes 0.25 mm of the earliest phase of the extension zone. (cf. the results obtained for onion roots by Wanner 1950). When comparing roots with different growth rates with each other, it is necessary to consider

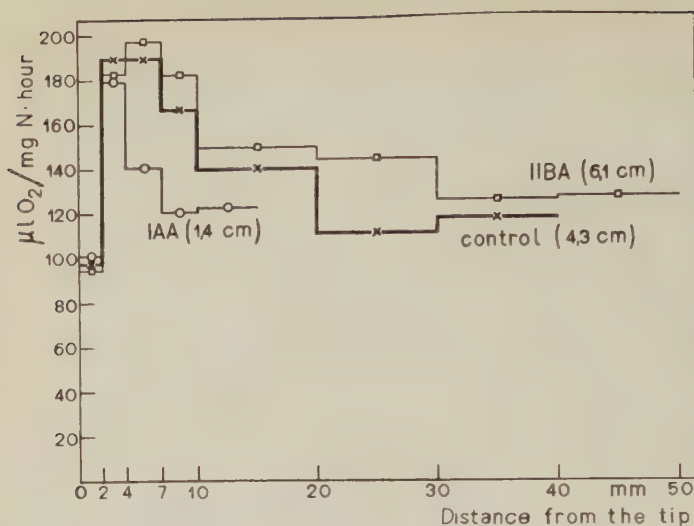


Figure 4. Oxygen uptake in root segments per mg total nitrogen. The same material as in figure 4. Each value is the mean of two or three experiments.

that the length of the extension zones varies (see table 1). It is seen from figure 4 that the difference between stimulated and inhibited roots does not imply a difference in the intensity of the respiration during the extension growth but a difference in the duration of the period of intense respiration. *The respiration rate will reach about the same level irrespectively of the growth rate of the roots or growth substances added.* Thus the respiratory increase in the beginning of the extension zone is not influenced at all or, in any case, very slightly by the growth regulators. However, the length of the period of high metabolic activity is determined by the length of the extension period. This means that the whole oxygen uptake *per unit protoplasm* from the beginning of the extension growth to an arbitrary time after the completion of this depends on the size of the elongation. An inhibition of the cell elongation will lead to a smaller oxygen uptake for the whole period as is shown by the diagram in figure 5. The consequence of this is that the energy, available for cell processes, to a certain degree will be proportional to the magnitude of the extension growth. This will account for the fact that the rate of synthesis of cell components is dependent upon how large the extension growth is (figure 1). The surplus of protoplasm, synthesized as a consequence of a more active metabolism, will in its turn contribute to a further increase in the respiration *of the whole root*. The differences in oxygen uptake for the pieces of the roots developed during the two days with different growth rates are thus mainly due to differences in the amount of protein synthesized (see table 2). The table shows that there is a close connection between respiration and nitrogen content independent of the growth rate. This is consistent

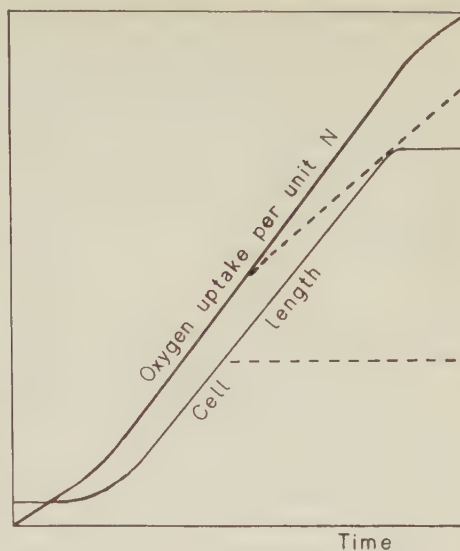


Figure 5. Diagrammatic representation of the connexion between respiration and growth of roots. The dashed lines show the case of growth inhibition.

with the fact that the relation between extending tissue — showing an intense respiration — and mature tissue will be about the same in roots with different growth rates, as is shown in table 1.

The results are consistent with the view that the differences in respiration are a consequence of the differences in cell elongation. There is no evidence for assuming that the increase in the respiration taking place in the extension zone is connected with the mechanism of action of the growth substances.

Discussion

The transition to the intense metabolic activity in the extension zone and the gradual regression to a lower activity after the completion of the extension growth is an interesting phenomenon with several general biological aspects. Strong changes between different intensity in the cell processes are characteristic of living cells on the whole. Particularly striking are such activity changes as the resumption of high metabolic activity after resting periods for example in the germination. Folkes, Willis, and Yemm (1952) demonstrated that the respiration in germinating barley, calculated as mg CO_2 per mg protein N, increased from zero to a maximum reached three days after the beginning of the germination. Then it decreased and was 8—10 days after the germination only 40 per cent of the maximum respiration. Such changes in the metabolic intensity owe certainly their occurrence to several co-operating factors, which

Table 2. *The oxygen uptake in the whole part of the root grown for two days excluding the meristem (the tip segment). The values have been obtained by adding the values of the different segments shown in figures 4 and 5.*

Growth substances	Root growth during two days (cm)	Oxygen uptake ($\mu\text{l O}_2/\text{hour}$)	
		per root	per mg N
Control	4.3	3.60	135
IAA	1.4	2.53	139
IIBA	6.1	4.22	143

influence the activity of the enzymes in the protoplasm. Which factors are concerned and how they act is imperfectly known, and a discussion of these problems must for this reason be to a large extent hypothetical.

If the ADP-supply is the limiting factor of respiration, an increased consumption of respiration energy by transfer of ATP to ADP will result in increased respiration (cf. Bonner and Bandurski 1952). A respiratory increase caused by this mechanism would, however, hardly continue in excised tissue, in which the growth has ceased owing to lack of substrate. The respiration of the extension zone is, as matter of fact, less sensitive to lack of substrate than the respiration of other parts of the root (Karlsson and Eliasson 1955). Nor are there any reasons for assuming that the great differences in the respiration discussed here are caused by differences in the amount of substrate supply. It is noteworthy, however, that the zone with the most intense oxygen uptake also is the zone with the most intense synthesis of cell components (cf. Kopp 1948). Nevertheless, it is hardly possible to deduce the causal connection between synthetic activity and respiration; they may be regarded, as different aspects of the same thing: the over-all metabolism.

Changes in the enzyme activity during the growth have been demonstrated for several enzymes such as dipeptidase, glycine oxidase, phosphatase, and invertase (Robinson and Brown 1952, 1954). Berger and Avery (1943) obtained an increase in the activity of alcohol and malic acid dehydrogenases parallel to an IAA-induced increase of the growth of *Avena* coleoptiles. Thus it is possible that an increase in the content of respiratory enzymes in the same amount of protoplasm may contribute to the respiratory increase obtained. Audus and Garrard (1953), as a matter of fact, assumed the increase in the respiration per section, obtained during growth in excised pea roots, to be caused by synthesis of respiratory enzymes during the extension growth.

The activity of a definite amount of enzyme is, however, also determined by the way in which the enzyme molecules are arranged in the protoplasm, i.e., by the structure of the protoplasm. Changes in this structure may for that reason result in changes in the enzyme activity. It may be that such changes, occurring during the cell extension, may cause the change of the metabolic activity of the protoplasm. The enlargement of the boundary areas of the protoplasm, the formation of vacuoles and of the tonoplast must involve considerable changes of the organization of the protoplasm. As many pro-

cesses in the living cell are assumed to occur at boundary layers, it should not be astonishing if the enlargement of these boundary areas, occurring when the compact protoplasm of the isodiametric embryonic cells extends to the thin layer along the walls of the elongated cells, resulted in a greater intensity of the cell processes. There may also be an influence by the changed diffusion conditions. It is noteworthy, however, that the high metabolic activity occurs simultaneously with the growth changes in the cells and then decreases to a lower level. Consequently, it is not possible to conclude that the differences between the embryonic and the elongated cells have any importance for the metabolic intensity of the protoplasm. This is consistent with the fact that, according to the experimental results shown in figure 4, the oxygen uptake is very slightly influenced by the cell length, in the mature root tissue.

It has been established that the oxidation processes in embryonic tissue are bound to the mitochondria (cf. Millerd 1953). Of great interest in this connection is the hypothesis that the tonoplast has a similar function in the elongated cells (Butler 1953). If the tonoplast during the course of the extension growth takes over the function of the mitochondria, this change ought to influence the intensity of the oxidation processes.

The condition of the protoplasm in different phases of development of the root may also be described as different degrees of lability or stability (cf. Bünning 1953). A high degree of lability implies a high sensitivity to different factors and an intense metabolism. The connection between the state of the protoplasm and the metabolic rate thus may be described in the following way. During the embryonic phase with the cells only slowly enlarging, the state of the protoplasm is rather stable and the metabolic rate moderate. During the violent increase of the cell size in the extension phase and the filling of a large part of the cell lumen with cell sap the protoplasm will enter a labile state involving a more intense metabolism. This intense metabolism does not cease at once, when the extension ends, but decreases only gradually as the stability of the protoplasm increases. Robinson and Brown (1954), who investigated how the activity of certain enzymes changes in excised sections of bean roots, also came to the result that the protoplasm became more labile during the extension.

It may be supposed that the stability of the protoplasm is closely connected with the state of the layers limiting the protoplasm towards the surrounding media, that is the tonoplast inwards to the vacuole and the plasmalemma outwards to the cell wall. These layers are usually considered to be rather well defined structures characterized by high lipid content (cf. Frey-Wyssling 1948). It seems probable that this stable quality develops only gradually and that the boundary layers during the extension are more undifferentiated. This may be the consequence if the processes, necessary for the formation

of the protoplasmic membranes, do not keep pace with the rapid increase in area during the extension. Thus the extension of the wall will bring the protoplasmic membranes into a labile state which will be abolished after the completion of the extension.

The condition of the outer protoplasmic membrane must be of great importance for the growth of the cell wall. The prerequisite of both softening of the cell wall and the intussusception of new wall material must be a close contact between protoplasm and cell wall. On that ground the hypothesis may be proposed that it is necessary for the plasmalemma to be labile to a certain extent in order that the cell wall growth may go on in a normal way. If the membrane is too stabile, the growth processes are inhibited, while, on the other hand too pronounced lability will result in such disturbances that the elongation is interrupted too early. Several investigations have shown that IAA influences the properties of the protoplasmic membranes. The literature in this field has recently been discussed by Tonzig and Trezzi (1954) and Anker (1953). Thus there is reason to consider that IAA influences the stability of the plasmalemma. The regulation of cell wall growth may tentatively be explained as the result of a combination of the effects exerted on the plasmalemma by the growth in area and by IAA.

Summary

Respiration measurements were carried out on segments of wheat roots cultured under sterile conditions. A simple method for sterile culturing is described. The extension growth of the roots was regulated by supplying a growth-inhibitory or a growth-stimulatory growth substance to the nutrient solution.

The connection between increase in length and increase in fresh weight, dry weight, and total nitrogen of the roots has been investigated. The results support the idea that it is the elongation that is primarily regulated by the growth substances and that differences in the other properties are a consequence of the differences in extension growth.

The earlier established fact that there is a considerable increase in respiration rate per unit nitrogen in the extension zone is confirmed, and some tentative suggestions as to the mechanism of it are discussed.

The experimental results show that the magnitude of the respiratory increase is independent of supplied growth substances but the duration of the period of intense respiration is due to the duration of the growth period. This constitutes a way in which the magnitude of the extension growth may influence the metabolic processes.

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References

- Almestrand, A.: Studies on the growth of isolated roots of barley and oats. — *Physiol. Plant.* 2: 372. 1949.
- Anker, L.: The effect of indole acetic acid and other growth promoting substances on the endogenous respiration of the *Avena* coleoptile. — *Acta Bot. Neerl.* 2: 22. 1953.
- Audus, L. J. & Garrard, A.: Studies on the growth and respiration of roots. I. The effects of stimulatory and inhibitory concentrations of β -indolylacetic acid on root sections of *Pisum sativum*. — *Journ. Exp. Bot.* 4: 330. 1953.
- Avery, G. S. Jr.: Stimulation of respiration in relation to growth. — *Plant Growth Substances*. Univ. Wisconsin Press, Madison, 1951.
- Baldovinos, G.: Reacciones respiratorias de los sistemas meristematicos de la raiz primaria de maiz. — *Rev. Soc. Mex. Hist. Nat.* 11: 77. 1950.
- Growth of the root tip. — *Growth and Differentiation in Plants*. The Iowa State College Press, Ames, Iowa, 1953.
- Berger, J. & Avery, G. S., Jr.: Action of synthetic auxins and inhibitors of the *Avena* coleoptile. — *Amer. Journ. Bot.* 30: 290. 1943.
- Bonner, J.: Relations of respiration and growth in the *Avena* coleoptile. — *Amer. Jour. Bot.* 36: 429. 1949.
- & Bandurski, R. S.: Studies of the physiology, pharmacology, and biochemistry of the auxins. — *Ann. Rev. Plant Physiol.* 3: 59. 1952.
- Bosemark, N. O.: The influence of nitrogen on root development. — *Physiol. Plant.* 7: 497. 1954.
- Brouwer, R.: The regulating influence of transpiration and suction tension on the water and salt uptake by the roots of intact *Vicia faba* plants. — Amsterdam 1954.
- Brown, R. & Broadbent, D.: The development of cells in the growing zones of the root. — *J. Exp. Bot.* 1: 249. 1950.
- Reith, W. S. & Robinson, E.: The mechanism of plant cell growth. — *Symp. Soc. Exp. Biol.* 6: 329. 1952.
- Burström, H.: Studies on growth and metabolism of roots. IV. Positive and negative auxin effects on cell elongation. — *Physiol. Plant.* 3: 277. 1950.
- Studies on growth and metabolism of roots. V. Cell elongation and dry matter content. — *Physiol. Plant.* 4: 199. 1951 a.
- Studies on growth and metabolism of roots. VI. The relative growth action of different isobutyric acid derivatives. — *Physiol. Plant.* 4: 470. 1951 b.
- Studies on growth and metabolism of roots. IX. Cell elongation and water absorption. — *Physiol. Plant.* 6: 262. 1953.
- Studies on growth and metabolism of roots. XI. The influence of auxin and coumarin derivatives on the cell wall. — *Physiol. Plant.* 7: 548. 1954.
- Butler, G. W.: The connexion between respiration and salt accumulation. I. Preliminary note. — *Physiol. Plant.* 6: 662. 1953.
- Bünning, E.: *Entwicklungs- und Bewegungsphysiologie der Pflanze*. 3:e Aufl. — Berlin, Göttingen, Heidelberg, 1953.
- Christiansen, G. S. & Thimann, K. V.: The metabolism of stem tissue during growth and its inhibition. II. Respiration and ether-soluble material. — *Arch. Biochem.* 26: 248. 1950.
- Folkes, B. F., Willis, A. J. & Yemm, E. W.: The respiration of barley plants. VII. The metabolism of nitrogen and respiration in seedlings. — *New. Phytol.* 51: 317. 1952

- Frey-Wyssling, A.: Submicroscopic morphology of protoplasm and its derivatives. — Elsevier Publ. Comp., New-York 1948.
- Goddard, D. R. & Meeuse, B. J. D.: Respiration of higher plants. — Ann. Rev. Plant Physiol. 1: 207. 1950.
- Gordon, S. A.: Physiology of hormone action. — Growth and Differentiation in Plants. The Iowa State College Press, Ames, Iowa, 1953.
- Hansen, B.: A physiological classification of »Shoot Auxins» and »Root Auxins». I. — Bot. Not. 230. 1954.
- Karlsson, B. & Eliasson, L.: The respiratory quotient in different parts of wheat roots in relation to growth. — In preparation.
- Klein, G.: Handbuch der Pflanzenanalyse, Band I. — Springer, Wien, 1931.
- Kopp, M.: Über das Sauerstoffbedürfnis wachsender Pflanzenzellen. — Ber. Schw. bot. Ges. 58: 283. 1948.
- Machlis, L.: The respiratory gradient in barley roots. — Amer. Journ. Bot. 31: 281. 1944.
- Millerd, A.: Respiratory oxidation of pyruvate by plant mitochondria. — Ach. Biochem. 42: 149. 1953.
- Robinson, E. & Brown, R.: The development of the enzyme complement in growing root cells. — Journ. Exp. Bot. 3: 356. 1952.
- — Enzyme changes in relation to cell growth in excised root tissues. — Journ. Exp. Bot. 5: 71. 1954.
- Tonzig, S. & Trezzi, F.: Ricerche sull'azione dell'acido indol-3-acetico. I. — Rend. Accad. Naz. Lincei, Ser. 8. 16: 603. 1954.
- Umbreit, W. W., Burris, R. H. & Stauffer, J. F.: Manometric techniques and tissue metabolism. — Burgess Publ. Co., Minneapolis. 1949.
- Wanner, H.: The zonal graduation of respiratory intensity in the root. — Ark. Bot. 31 A, 9: 1—9. 1944.
- Wanner, H.: Histologische und physiologische Gradienten in der Wurzelspitze. — Ber. Schw. bot.-Ges. 60: 404. 1950.

The Conversion of Protochlorophyll to Chlorophyll a in Continuous and Intermittent Light

By

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Introduction

Smith and Benitez (8) — using absorption spectrophotometric methods for the pigment determination — found that the conversion of protochlorophyll to chlorophyll a is a second order reaction. The temperature dependence of the reaction, however, is low. At -195° C no transformation occurs, but even at -70° C fairly rapid conversion takes place which increases in rate and extent with increase in temperature up to 40° C. At higher temperatures no reaction occurs. This is probably due to the destruction of the pigment-protein complex, the holochrome, cf. Smith and Young (9). From these findings the conclusion was drawn »that the formation of chlorophyll from protochlorophyll is not strictly a photochemical intramolecular transfer of hydrogen within the holochrome but requires collision processes as well» (Smith and Benitez, 8, p. 142). According to our present knowledge this reaction consists in adding two hydrogen atoms to protochlorophyll in position 7 and 8, to form chlorophyll a. The mechanism involved to account for a second order reaction is not known.

One way of finding out whether we have non-photochemical reactions of relatively long duration contributing to the conversion process would be to study the reaction under the influence of intermittent light. It could be anti-

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culated that the conversion yield obtained in flashing light would be different as compared to that in continuous illumination if the reaction involves several stages, some of them not being strictly photochemical. It is well known that the photosynthetic yield per unit of light absorbed is increased if the light is administered as short flashes followed by considerably longer dark periods. From such experiments it could be proved that the first steps in photosynthesis involve a very rapid photochemical reaction accompanied by a slower light-independent chemical reaction (Emerson and Arnold, 1).

The methods for determination of pigments are mainly based on absorption spectrophotometry. These methods, when used for determination of protochlorophyll, require comparatively large amounts of material due to low concentration of pigment. They are also rather time consuming as partition between solvents after the extraction must be done before quantitative determination is possible. In flashing light experiments many series of illuminations are required. It would therefore be advantageous to find another property of the pigment which could be used for a rapid determination of the concentration. Such a property we have in the fluorescence.

Chlorophyll as well as its precursor protochlorophyll both show a strong fluorescence. It is therefore possible to use this property for quantitative determination of the pigments present. Within limits the fluorescence emitted by each pigment is proportional to the pigments' concentration and is independent of accompanying substances. Only if the absorption maxima of such an impurity coincides with a maximum in the fluorescence spectrum, there is considerable reabsorption of the fluorescence. If there is constant absorption this can be easily allowed for, but if there is changing absorption then the corrections are difficult. This happens when chlorophyll *a* is formed. There is an increasing absorption of protochlorophyll fluorescence by chlorophyll *a* as it is produced. Since the main fluorescence peaks of protochlorophyll and chlorophyll *a* are separated by 42 m μ , each of these pigments can be determined quantitatively in the presence of the other by spectrofluorimetric methods. The amount of material required to make a quantitative determination of a fluorescent substance is considerably smaller than the amount necessary if absorption spectrophotometry is used for the same purpose. From a practical point of view, this means that the amount of pigment present in a small fraction of a leaf is enough for such a determination. With absorption methods several leaves have to be extracted in order to obtain concentrated enough solutions.

In experiments with flashing light the accuracy required for the determination of the pigments is rather high because the difference between two series may be small but yet significant.

The first part of this paper deals therefore with a quantitative study of

the conversion of protochlorophyll to chlorophylla by means of spectrofluorimetric analysis in order to check the accuracy of the method. Since spectrophotometric methods demonstrated the near quantitative conversion of protochlorophyll to chlorophylla (Smith and Benitez, 8), this conversion was used to standardize the spectrofluorimetric method. After such a standardization the spectrofluorimetric method give nearly as consistent and accurate results for the ratio of the two pigments during their conversion as did the spectrophotometric method of analysis.

Technique

Plant material

Etiolated leaves of barley were used for these experiments. The strain was the one used in earlier experiments by Smith and Benitez, 8. Seeds were soaked in tap water for 24 hours and thereafter planted in moist sand and kept in complete darkness in a thermostat at 22° C for eight days. The leaves had then reached a length of about 10 centimeters and could be used for the experiments. For the short times when the pots were watered and during cutting, the leaves were illuminated with very weak green light obtained by means of an interference filter with a maximum transmission at 500 mμ.

Light source and filters

A General Electric projection lamp, 115 V; 180 watt, was used for causing the transformation. The lamp was operated with current from a voltage stabilizer. The light was passed through an interference filter with maximum transmission at 660 mμ and a half bandwidth of 13.1 mμ. This light lies within the red band of the action spectrum for the chlorophyll formation (Koski, French and Smith, 5). The intensity of the light was measured by means of a thermopile coupled to a mirror galvanometer.

Measurement of the fluorescence

The apparatus used for fluorescence measurements is described in earlier papers. (French and Young, 3; Virgin, 10).

When intact leaves were analyzed after different degrees of conversion had been produced, they were first infiltrated with water by boiling — see below — and pressed between two pieces of glass so that no air-filled spaces were left (cf. Virgin, 10). The leaves were placed in the sample holder so that the beam of exciting light was kept within the leaf area. When the fluorescence of glycerol suspensions was measured, a Beckman absorption cell was used. The front surface of the cell was covered with an aluminum foil containing an opening equal in size to that of the entrance slit of the analyzing monochromator.

The light for exciting fluorescence was the 436 mμ mercury line, isolated by means of Corning filter No. 3389, 5113, and 4305.

In order to insure the constancy of the exciting light during any series of measurements, the light from a low-intensity neon lamp — this as well as the mercury lamp operated with stabilized voltage — could be made to fall on the photocell. Any change in the response could thus be found. The intensity of the light had to be extremely low and could be changed for different settings of the amplification of the photo-current, as the light was first reflected from a black-painted surface before entering the photocell. By changing the angle of this black surface the amount of light could be given any value desired.

Calculation of pigment concentration from fluorescence spectra

The fluorescence spectra of protochlorophyll and of chlorophyll a in heat-killed leaves are shown in Figure 1. As shown, the fluorescence spectrum of protochlorophyll has its main peak at 636 mμ and that of the conversion product, chlorophyll a, at 678 mμ. The height of any part of the spectrum of either single pigment can be used as a measure of the concentration of the pigment, if the comparison is made at the same wave length on different occasions, provided no reabsorption takes place. From a practical point of view, however, the value for the main peaks is most useful.

A mixture of the two pigments will show a fluorescence spectrum in which the two single spectra are present in intensities proportional to the fractions of the respective pigments in the mixture. In order to be able to calculate the percentage conversion of protochlorophyll to chlorophyll a after different periods of illumination, one must therefore know the spectral curve for each pigment under the experimental conditions used.

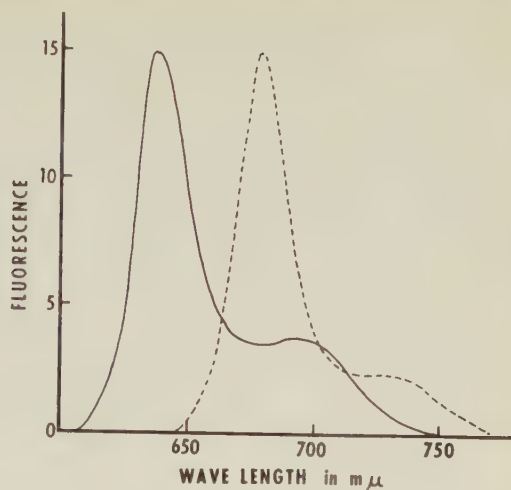
Calculation of the decrease in protochlorophyll

The height of the peak at 636 mμ in the fluorescence spectrum of plant material which has not been exposed to light is taken as a measure of the initial amount of protochlorophyll. After illumination this peak will decrease as more and more protochlorophyll is transformed into chlorophyll a. In this case the presence of chlorophyll a will not interfere with the measurement of protochlorophyll, since the fluorescence of chlorophyll a is negligible at this wave length. The value for the protochlorophyll fluorescence must be corrected, however, for the amount of protochlorophyll which is inactive and will not be transformed — see below.

The percentage of the total decrease of protochlorophyll will thus be:

$$\frac{\text{Decrease in fluorescence intensity at 636 m}\mu}{\text{Initial fl. intens. at 636 m}\mu - \text{Intensity of inact. protochlorophyll}} \times 100$$

Figure 1. *Relative fluorescence-spectrum curves of protochlorophyll (—) and chlorophyll a (---) in heat-killed leaves of barley. The height of the principal bands are arbitrarily adjusted to equality.*



Calculation of the increase in chlorophyll a

From the measurements of the fluorescence of leaves containing only protochlorophyll — unexposed etiolated leaves — the following ratio can be calculated:

$$\frac{\text{Fluorescence intensity at } 636 \text{ m}\mu}{\text{Fluorescence intensity at } 678 \text{ m}\mu} \quad (\text{ratio } 1)$$

This ratio is always constant for all fluorescence spectra of protochlorophyll if the concentrations are held at such low values that no reabsorption of fluorescent light takes place.

If the fluorescence of protochlorophyll at 636 mμ is known, the fluorescence due to protochlorophyll at 678 mμ can be calculated.

The intensity of the chlorophyll a fluorescence in an illuminated leaf will be the value for the observed intensity at 678 mμ (the main peak of chlorophyll a) minus the quotient of the value for the fluorescence intensity at 636 mμ and the ratio 1. The fluorescence intensity of chlorophyll a will thus be:

$$\text{Observed intensity at } 678 \text{ m}\mu - \frac{\text{Intensity at } 636 \text{ m}\mu}{\text{ratio } 1}$$

In order to be able to calculate the percentage of chlorophyll a formed, one has to know the value for the intensity of chlorophyll a fluorescence after complete transformation. The ratio of protochlorophyll fluorescence at the very beginning of the conversion to the chlorophyll a fluorescence after complete transformation will be a constant which varies with the wave

length of the exciting light. When this constant has been determined it can be used to calculate the end value for the chlorophyll a fluorescence corresponding to any amount protochlorophyll.

Complete transformation is obtained after about 10 minutes illumination by a 50 watt incandescent lamp at a distance of 50 centimeters. By »complete transformation» is meant a conversion of about 90—95 per cent. It has never been possible to get the initially present protochlorophyll to disappear completely. The reason for this is not known but may be due either to the presence of a certain amount of injured cells where the metabolic processes have ceased or to a partial light-destruction of the conversion mechanism (cf. Virgin, 11).

From the values for the chlorophyll a fluorescence after complete transformation the percentage of chlorophyll a formed can be calculated.

If protochlorophyll is the precursor of chlorophyll a, the chlorophyll a formed, calculated as percentage of the values after complete transformation, should be the same as the amount of protochlorophyll which has disappeared, calculated as percentage of the amount initially present.

Experimental

Experiments with intact leaves, continuous illumination

For the illumination experiments leaves were cut from the plants and transferred to a closed glass bottle kept in a light proof metal container. From the leaves a piece, 2 cm long, was cut 2.5 cm below the apex. As the distribution of protochlorophyll in the leaf differs very much from the apex to the base (Figure 2), it is of importance to take pieces from the same relative position in order to keep the pigment concentration about the same from one experiment to another.

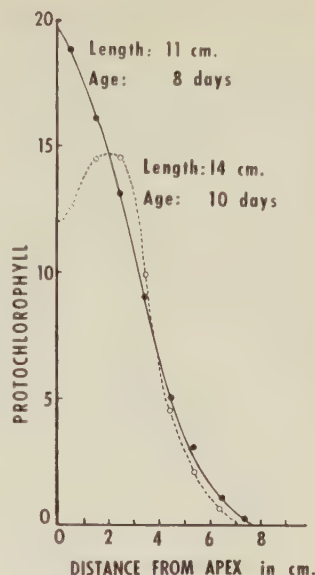
The 2 cm long piece of leaf was cut in two equal parts, parallel to the long axis. The fluorescence of these two parts shows identical values (Figure 3). Thanks to this finding one half of the leaf could be used for illumination experiments, the other as control.

All the manipulations with the leaves were performed in very weak green light (flash light bulb plus interference filter, 500 mμ). After illumination the leaves were dipped for 6 seconds in boiling water which instantly stopped the protochlorophyll transformation, without any pigment loss from the tissue. The measurement of fluorescence was performed not later than two hours after illumination and heating. In the meantime, the killed leaves were kept in darkness in a refrigerator.

During illumination the piece of leaf was slightly pressed between two glass plates in a holder. The latter could be replaced by a photocell at the position occupied by the leaf sample. The intensity of the light varied from 32.4 to 33.0 ergs/cm.² sec. during the different experimental series.

The results from two series of experiments are shown in Figure 4 and Table 1. From the values in the Table it is obvious that the variation in the

Figure 2. *Distribution of protochlorophyll in etiolated leaves of barley.* The curves represent measurements of two single leaves. The length can vary considerably from one leaf to another and also the pigment distribution in the top part of the leaves. The general trend, however, is a shift of the maximum concentration of pigment to parts some centimeters below the apex as the leaves grow older. It looks from preliminary experiments as if the protochlorophyll in the top parts of older leaves does not transform as easily as that from lower parts. This may account for the amount of «inactive» protochlorophyll in most of the experiments. The points in the Figure represent the average fluorescence intensity for one centimeter long pieces of the leaf.



material is fairly great. Average values from ten single series gave slightly better agreement between percentage decrease of protochlorophyll and formation of chlorophyll a, but the deviations from the theoretical values are still comparatively large. These deviations are mainly due to two factors. It is extremely important that the leaves be completely infiltrated before the measurement of the fluorescence. Due to light scattering even the slightest presence of air-filled intercellular spaces will give a decrease of the intensity of fluorescence at the main peak of chlorophyll a and probably even of protochlorophyll. At the same time the second peaks increase in height (Virgin, 10). In these experiments great care was taken to get complete infiltration, but it is still possible that small air-filled spaces could have been present occasionally.

It is also possible that some deviations are caused by using very small pieces of leaf. Local variations in leaf thickness or physiological anatomy would be particularly obvious when using small pieces from different leaves. That the deviations are actually due to variations in the plant material and not to instrumental error can be seen from experiments with glycerol suspensions of leaf homogenates described below.

It must be emphasized here, however, that the amount of protochlorophyll which is present in the small leaf sample used for a measurement is only $8 \cdot 10^{-5}$ mg., (calculated from data given for etiolated barley by Smith, 6). This amount of pigment is far below what can be measured by means of absorption spectrophotometry.

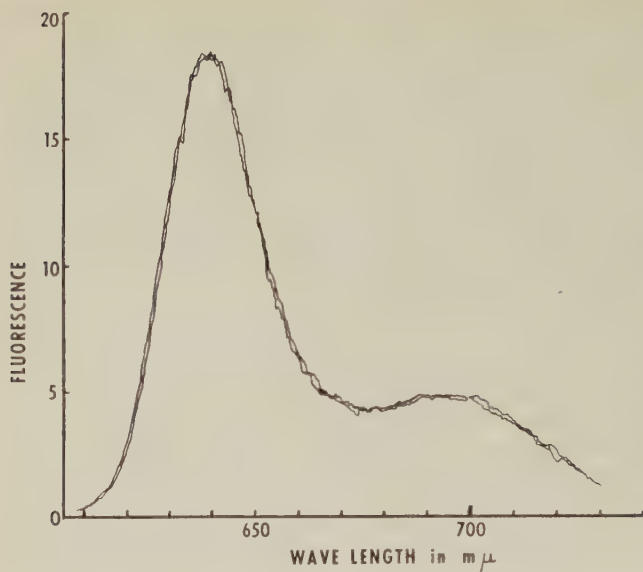


Figure 3. *Fluorescence of two halves of a leaf, 2.5 cm below apex. The curves shown are the actual records as they appear on the paper of the recorder without any smoothing of the curve lines. They are practically identical which shows that the pigment distribution in a leaf is very even laterally.*

*Experiments with glycerol suspensions of leaf homogenates,
continuous illumination*

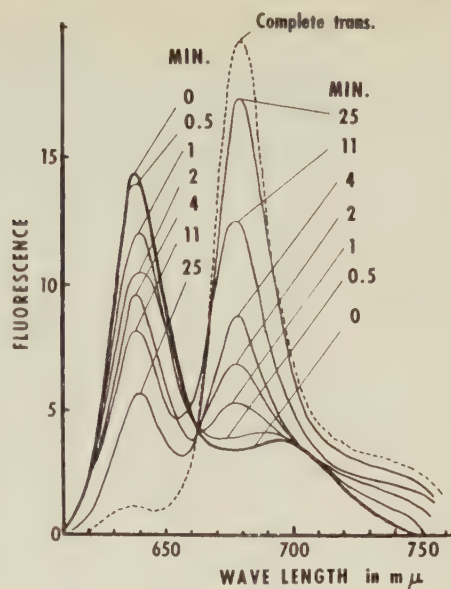
Light-induced transformation of protochlorophyll to chlorophyll *a* is not restricted to undamaged leaves or to chloroplasts. If etiolated leaves are ground in water-free glycerol or concentrated sugar solution in darkness and the suspension thus obtained is centrifuged or filtered, a slightly yellowish opalescent liquid is obtained. This liquid gives at first the absorption spectrum of protochlorophyll, but after some seconds of exposure to light, the bands of chlorophyll *a* become visible (Smith and Benitez, 7). A microscopical examination of the liquid shows that only very small fragments of the chloroplast material are present. The suspension retains its activity for more than a week if kept in a refrigerator but loses the activity within a day or two at room temperature. The decrease in activity is due partly to an increase in the fraction of protochlorophyll which will not transform and partly to a decrease in the rate of transformation.

When working with glycerol suspensions the following procedure was followed:

About 25 g of etiolated barley leaves were ground with 75 ml glycerol in darkness until a homogeneous brei was obtained. In the first experiments this grinding was done in a mortar by hand but later on by means of a Waring blender. The brei was then pressed through muslin, collected in a Pyrex flask and immediately cooled.

For the illumination experiments, 5 ml of the thick suspension was poured into

Figure 4. Fluorescence spectrum of leaves of etiolated barley after illumination for different periods of time. In a series of measurements the curve representing zero illumination of each leaf was adjusted to a standard height at the fluorescence maximum. This adjustment factor was then applied to each curve representing measurement of the same leaf after illumination. In the Figure one unit for the fluorescence equals a concentration of about 0.0001 per cent of protochlorophyll.



a crystallizing dish, 5 cm in diameter. After the temperature became constant the dish was illuminated from above with the same light as was used in the experiments with intact leaves. The dish could be replaced by a thermopile. After illumination the suspension was poured into a test tube which was kept in boiling water for 1.5 minutes during which time the temperature in the suspension rose to 90° C, warm enough to stop conversion immediately. The suspension was then rapidly cooled and kept in a refrigerator until time for the fluorescence measurement. The same cell was used for all fluorescence determinations within one series of experiments. Between the measurement of different suspensions the cell was thoroughly rinsed with distilled water and dried.

Table 1. Change in pigment concentration after illumination of leaves of etiolated barley.

Ill. time Min.	Protochlorophyll		Chlorophyll a	
	Conc. ¹	Decrease in % of initial conc.	Conc. ¹	Increase in % of conc. after compl. trans. ²
0	13	0	0	0
0.5	12.7	2.3	0.7	3.6
1	11.8	9.3	2.5	13.1
2	9.2	29.2	4.3	22.6
4	8.3	36.2	6.4	33.7
11	6.9	47.0	10.4	54.8
25	4.5	65.4	15.8	85.0
∞	0	100	19	100

¹ One unit represents a concentration of about 10^{-5} mg of pigment per 1 mg of leaf.

² Corrected for »inactive» protochlorophyll.

Table 2. *Change in pigment concentration after illumination, of glycerol suspensions of etiolated barley leaves.*

Ill. time Sec.	Experiment I		Experiment II		Experiment III		Experiment IV	
	Decr. of protochl. in % of init. conc.	Incr. of chloroph. in % of conc. after compl. transf.	Decr. of protochl. in % of init. conc.	Incr. of chloroph. in % of conc. after compl. transf.	Decr. of protochl. in % of init. conc.	Incr. of chloroph. in % of conc. after compl. transf.	Decr. of protochl. in % of init. conc.	Incr. of chloroph. in % of conc. after compl. transf.
0	0	0	0	0	0	0	0	0
15	—	—	25.0	24.6	18.9	22.8	8.55	4.47
30	23.9	20.7	40.2	42.6	34.8	38.9	12.8	10.8
60	39.1	38.7	59.6	57.2	52.1	59.7	23.9	19.7
120	47.8	56.0	70.8	70.0	68.6	68.7	36.7	35.4
240	71.7	70.7	64.3	68.6	76.3	73.0	51.2	53.2
360	84.8	71.3	77.8	78.3	76.7	74.5	62.7	59.7
480	89.2	70.7	75.5	73.7	76.8	75.7	69.2	68.5
660	89.2	73.0	78.3	78.4	78.6	77.0	74.4	75.0
900	89.2	69.8	79.8	78.4	80.2	78.3	85.0	80.2
1200	91.3	64.2	89.7	82.5	91.4	81.6	89.7	85.5
1500	95.6	55.7	94.7	81.2	93.7	86.0	91.4	86.5

The results from four different experimental series are shown in Table 2. The intensity of the light was 129 ergs/cm.²·sec. in Experiment No. I; 108 ergs/cm.²·sec. in Experiment No. II and III, and 64.8 ergs/cm.²·sec. in Experiment No. IV.

The percentage of decrease and increase of pigments in the Table is calculated by assuming that the amount of inactive protochlorophyll remained constant. In the glycerol suspension there is a considerable amount of inactive protochlorophyll which will never transform. If the amount of protochlorophyll transformed is calculated as a percent of the total amount initially present, we will thus get misleading values. The end values for the transformation of active protochlorophyll must be known in order to calculate the conversion values. Assuming that the conversion follows a second order reaction as has been found by Smith and Benitez (8), the end values for the protochlorophyll were calculated for different periods of illumination according to the formula for a second order reaction:

$$T_{\infty} = \frac{T' \cdot \frac{T''}{t''} \cdot (t'' - t')}{T' - \frac{T''}{t'} \cdot t'}$$

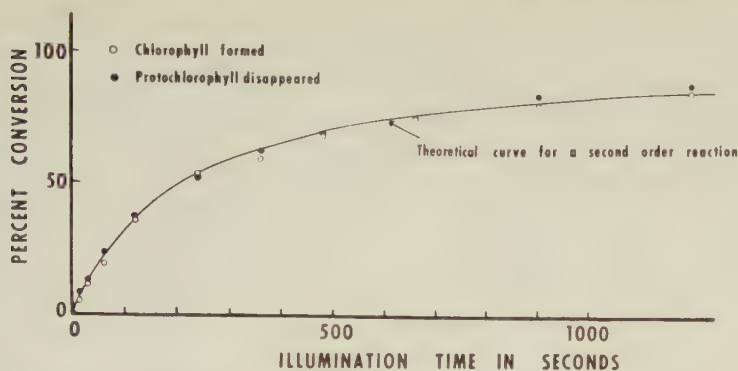


Figure 5. Conversion of protochlorophyll to chlorophyll a in etiolated barley leaves during different periods of illumination. The dots represent experimental results; the line represent theoretical conversion by a second order reaction with a constant giving the best fit with the experimental values.

where

T_{∞} = percent conversion at the end of the reaction

T' = » » after time t'

T'' = » » after time t''

The end values thus obtained were used as 100 per cent conversion in the calculations (Table 2) of the percentage conversion after different periods of illumination.

A study of Table 2 shows that at high intensities of light (Experiment No. I) a considerable light-destruction of chlorophyll a takes place. Illumination for more than 11 minutes decreases the chlorophyll a content gradually, resulting in an increasing difference between protochlorophyll disappeared and chlorophyll a formed. In the other experiments the quantities of the two pigments correspond fairly well, except for the slight destruction of chlorophyll a near the end of Experiment II. The experimental values agree well with the values for a theoretical second order reaction. This can be seen in Figure 5. The values from Experiment No. IV are here plotted together with a »second order curve» whose constant has been adjusted to match the experimental points as closely as possible. The curve was obtained by means of the curve analyzer designed by French et al. (4).

From these experiments the conclusion can be drawn that the method of using the fluorescence of pigments as a measure of their concentration under certain conditions can give as accurate results as the method of absorption spectrophotometry. The largest error in the determinations is to

Table 3. *Pigment conversion in continuous and intermittent light. Light intensity: 43 ergs/cm.² sec.*

Temp. ° C	Ill. time Min.	Contin. illum.	Intermittent illumination		
		Conversion %	Light periods Millisec.	Dark periods Millisec.	Conversion %
20	30	14.4	3.32	29.68	11.2
20	60	22.6	3.32	29.68	26.7
20	120	50.2	3.32	29.68	48.9
0	30	12.9	3.32	13.18	15.8
0	60	31.6	3.32	13.18	30.4
0	120	45.5	3.32	13.18	45.6
0	30	20.3	3.32	29.68	14.8
0	60	39.0	3.32	29.68	26.0
0	120	50.3	3.32	29.68	45.6
0	30	14.7	3.32	63.3	16.1
0	60	25.2	3.32	63.3	25.2
0	120	43.0	3.32	63.3	42.0

be found in biological variability of the material and not in the experimental procedure or instruments used.

Experiments with glycerol suspensions, intermittent illumination

In these experiments the percentage conversion in continuous light was compared with that obtained when the same amount of light — constant intensity — was given as short flashes followed by dark periods of different length.

The intermittent light was obtained by use of a rotating sector disc with openings of variable size. Rotation at any speed between 0 and 1800 r.p.m. was provided by a stepless variable speed transmission. In order to get light flashes of constant intensity without any intermediate intensity between light and dark, the image of the lamp was focused at the plane of the sector disc by a system of lenses. The single-filament lamp was adjusted so that the filament was parallel to the sides of the openings in the disc. By rotating the disc, the light beam was thus cut abruptly without any intermediate steps in light intensity.

Two series of experiments will be reported here. In one series the length of the light periods were kept constant at 3.3 milliseconds and the dark periods were varied from 13.18 to 63.3 milliseconds. This variation was obtained by changing both the size of the openings in the disc and the speed of the rotation. In the other series the light periods as well as the dark periods were changed by varying only the speed of the sector.

Table 4. *Pigment conversion with varying regimes of light and darkness. Light intensity: 43 ergs/cm.² sec. Temp.: 0° C.*

Ill. time	Sec.	Light periods	Millisec.	Dark periods	Millisec.	Conversion %
596		5.8		51.3		31.8
596		8.6		77.3		30.8
596		21		179		33.3
596		41		369		30.7
596		100		899		32.8
596		310		2690		29.2
596		1240		10760		31.2

For the studies of the pigment conversion glycerol suspensions of etiolated barley leaves were used and were prepared as in the preceding experiments.

The results of the experiments are shown in Tables 3 and 4.

In the experiments reported in Table 3 three points on the conversion curve were taken. The figures given are the percentage transformed protochlorophyll based on the amount initially present. No correction was made for inactive pigment.

In Table 4 the value for product of intensity \times time has been chosen so that we are on the steep part of the conversion curve. Any change in conversion rate would therefore have easily been detected.

It is obvious from the Tables that there is no significant difference in conversion yield between the series where continuous light was used as compared to the series with intermittent light. Even at 0° C we get the same values for the two series. Using the same reasoning as for the experiments with intermittent light for photosynthesis (Emerson and Arnold, 1), this means that no dark reaction is involved or that the half life time of substances formed is very short.

Discussion

The conversion of protochlorophyll into chlorophyll a shows several rather peculiar characteristic features. In the penetrating study by Smith and Benitez (8), the possibilities of different pathways have been discussed. The experimental data, especially the low temperature dependence within certain limits, point to a photochemical reaction being the main part of the process. Here, we have an anomaly in that transformation does not occur at temperatures as low as -195° C, as it should if the reaction were strictly photochemical. Also the fact that the reaction — even within the temperature limits where the temperature dependence is very low — is strictly bimolecular, shows that some non-photochemical reaction might be involved.

Taking into account the small chemical difference between the two pigment molecules involved, it is rather difficult, however, to make a scheme for the occurrence of a bimolecular reaction. Smith and Benitez (8) present two possibilities. Either one protochlorophyll molecule could react with another, but excited, molecule of the same kind or with a photodissociated product of the protochlorophyll holochrome (p. 142). In either case for the reaction to conform to the second-order rate law the rate of loss of excitation energy should be great as compared to the rate of the collision process.

The findings of this paper show that the yields per unit of irradiation are equal for continuous and intermittent illumination under the conditions used. They demonstrate that the conversion of protochlorophyll to chlorophyll *a* is not accompanied by a non-photochemical reaction of long life. Since dark periods as short as 13.18 milliseconds have no effect on the yield per unit of irradiation, it can be reasonably assumed that the half life of any species produced by the light is less than about one tenth of this value or about 1 millisecond. This indicates that whatever intermediate is formed by photochemical action is short-lived, for example, an activated molecular species or even a free radical (cf. Commoner et al., 2).

If higher intensities of irradiation could have been used, it is conceivable that an intermittency effect might have been found. But high light intensities bleach the pigments and their use would have jeopardized the experimental results. It is not known whether a saturation intensity exists for this reaction.

Summary

The conversion of protochlorophyll to chlorophyll *a* in intact barley leaves as well as in glycerol suspension of ground leaves has been studied by means of spectrofluorimetry.

By the use of fluorescence instead of absorption for determination of pigment concentration a much smaller amount of material is sufficient. The determinations can also be performed without isolation and purification of the pigments.

The results confirm the findings by Smith and Benitez (8) that the reaction is bimolecular.

Intermittent light did not increase the yield, which means — as the process follows the second order law — that this process has a half life time considerably shorter than the dark period used. The results do not exclude, however, the possibility of the existence of a saturation intensity above which conversion in intermittent light might increase the yield.

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References

1. Emerson, R. & Arnold, W.: A separation of the reactions in photosynthesis by means of intermittent light. — *J. Gen. Physiol.* 15: 391. 1932.
2. Commoner, Barry, Townsend, Jonathan & Pake, George E.: Free radicals in biological materials. — *Nature* 174: 689. 1954.
3. French, C. S. & Young, Violet K.: The fluorescence spectra of red algae and the transfer of energy from phycoerythrin to phycocyanin and chlorophyll. — *J. Gen. Physiol.* 35: 873. 1952.
4. — Towner, George H., Bellis, Donald R., Cook, Richard M., Fair, William R. & Holt, Walter W.: A curve analyzer and general purpose graphical computer. — *Rev. Sci. Instr.* 25: 765. 1954.
5. Koski, Violet M., French, C. S. & Smith, James H. C.: The action spectrum for the transformation of protochlorophyll to chlorophyll a in normal and albino corn seedlings. — *Arch. Biochem. and Biophys.* 31: 1. 1950.
6. Smith, James H. C.: Organic compounds of magnesium and phosphorus in relation to chlorophyll formation. — *J.A.C.S.* 69: 1492. 1947.
7. — & Benitez, Allen: the protochlorophyll-chlorophyll transformation: the nature of protochlorophyll in leaves. — *Carnegie Inst. of Wash. Year Book* 52: 149. 1953.
8. — & Benitez, Allen: The effect of temperature on the conversion of protochlorophyll to chlorophyll a in etiolated barley leaves. — *Plant Physiol.* 29: 135. 1954.
9. — & Young, Violet, M. K.: Chlorophyll formation and accumulation in plants. — *Radiation Biology*. Alexander Hollaender, Ed. (In press).
10. Virgin, Hemming I.: The distortion of fluorescence spectra in leaves by light scattering and its reduction by infiltration. — *Physiol. Plant.* 7: 560. 1954.
11. — Protochlorophyll formation and greening in etiolated barley leaves. — *Physiol. Plant.* 8. (In prep.).

Relation between Leaf Number and Ear Development in Spring-sown Barley and Oats

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Methods for determining vegetative and reproductive stages during the development of the shoot apex in barley and oats have been described in an earlier paper (Andersen 1). It was also investigated whether the occurrence of reproductive stages was correlated with the leaf number of the plants. Such a correlation was demonstrated, but full agreement was not found in all cases. In this paper an attempt is made to point out some of the factors which might promote or retard the transition from vegetative to reproductive growth.

The influence of seeding rate, soil fertility, and date of seeding are studied in field experiments, and also differences between varieties are studied in the field. In pot experiments, the effect of soil moisture and light intensity are tried. The effect of variation in day-length was tried, but in a small experiment only. Day-length in Denmark is presumably sufficient for a quick development of the ear. The emergence seldom takes place before April 15th, and at that time the day-length is 15 hours (from half an hour before sunrise to half an hour after sunset).

Some varieties of barley and oats are winter forms, which need a period of low temperature. The varieties used in the present and the earlier investigation have no cold requirement, but are true spring types. They head, if sown in June, when the average temperature is about 13° C. This does not necessarily mean that low or high temperature during emergence and early growth would not affect the relation between leaf number and ear develop-

ment. Some experiments were made in greenhouses with different temperatures, but the facilities were insufficient, and the results will not be reported.

The effect of temperature on the development of the young barley spike was studied by Borthwick, Parker and Heinze (3), and Hamilton (6) studied the effect on the oat panicle. Unfortunately, none of these authors counted the number of leaves. Borthwick, Parker and Heinze found that the development of the spike was delayed by low temperature (2 and 7° C), but favoured by higher temperature (18° C). Hamilton, however, found that excessively high temperature (28° C), prevents spikelet formation in oats.

In the earlier and the present investigation only the main shoot of the plants is examined. The ears development in the tillers is not considered, and the leaves on the tillers are not included in determining number of leaves. For a long time the number of tillers was considered unimportant, but a small experiment in 1952 showed that the ear in the main shoot was at the most advanced stage in plants without tillers. The number of plants with tillers has therefore been counted in all samples, and the relation between ear development and leaf number was found to be correlated with the degree of tillering. Where many tillered plants were found, the change from vegetative to reproductive growth was delayed until the leaf number was high.

Mitchell (8) working with rye grass, *Lolium perenne*, found that the number of tillers increased by increasing light intensity or by lowering the temperature. Mitchell claimed, as earlier suggested by Leopold (7), that variations in amount of tillering in grasses are associated with equivalent variations in rate of auxin production or sensitivity of the plant tissue to auxin. Gustafson (5), who investigated the auxin concentration in a number of plants found a low concentration in plants grown a) on a nutrition-deficient soil, b) at low temperature, and c) under high light intensity. Since a low auxin concentration is expected to cause formation of many tillers, there is good agreement between Gustafson's and Mitchell's results.

Gardner (4) gives an account of literature on tillering, and has made experiments himself. The formation of side shoots seemed to be benefited by a soil rich in mineral nutrients and with a suitable moisture content.

Methods

Some of the experiments were made in the field, and some were made in wooden boxes or as pot-experiments. The field experiments were laid out with 2-4 replications, and at the dates of analysis, 15 to 30 plants were taken at random from each plot. In the pot-experiments, 25 plants were grown in each pot, and all plants from 2-3 pots were examined.

The plants from each treatment were bulked, and the leaf number and ear development were determined by the method earlier described (Andersen 1). According to this method, the number of visible leaves were counted on the main shoot of each

plant and an average figure is calculated for 50 plants. Thereafter large and small plants were discarded, and 15 plants of average size selected for ear analysis. By this selection, care was taken that the percentage of plants with tillers was equal to the percentage in the whole sample.

The growing points or ears were classified according to their morphological development. The development stage was determined in each of the 15 plants, and an average figure calculated for the whole sample. In barley 13 development stages were used, and in oats 10. Those stages represent the whole development from emergence to heading. Most important is the change from stage 3 to 4 in barley and from stage 2 to 3 in oats. When stage 4 is reached in barley, spikelet primordia are visible, and the growing point has been transformed into an ear. In oats, reproductive growth is indicated in stage 3 by formation of branches of second order.

In most cases, samples were taken only when a definite leaf number was reached. All the plots in an experiment were not necessarily investigated on the same day, but analyses were made when the stage of development was found suitable for the purpose. That was done in order to make a comparison between different treatments more easy. Barley was analysed when the leaf number was 2.50, 3.50, and 4.50, i.e., the first analysis was made when 50 per cent of all the plants in a treatment had 2 leaves and the rest 3 leaves; the second analysis was made when 50 per cent had 3 leaves and the rest 4, and so on. Oats were analysed when the leaf number figured in the same way, was 3.50, 4.50, and 5.50.

It might perhaps seem more reasonable to make the examinations when the leaf numbers came close to whole figures, for instance 3.0 instead of 3.5. This, however, would be an inappropriate procedure since most of the plants develop new leaves at nearly the same time. When the average number of leaves lies close to a whole number it would, in many cases, increase very little for 2—3 days (Figure 1). Since the ear develops continuously, examinations at this stage could not show any close correlation between leaf number and ear development. Two samples, taken with 2 days interval in the same plot, would show approximately the same number of leaves, but different stages of ear. Samples where the leaf number were half-way between two whole figures could, as a rule, only be found on one single day.

Since it was not always possible to analyse when the leaf number was exactly —.50 (the dash before the point means 2, 3, 4, or 5), the figures indicating ear development were corrected and changed to the assumed value if the leaf number had been exactly —.50. Such correction was applied in experiments comprising a large number of analyses. In experiments where corrections were judged to be of minor importance, samples from all treatments were taken on the same day. This applies to the experiments described in tables 1 and 2, 8, and 9.

Method for correction figures for ear development when the leaf number was not exactly —.50. The correction figures were found by means of 33 pairs of analyses. Each pair was taken in the same plots, but with 2 days interval, so that the leaf number in the first was somewhat below —.50 and in the second above. Such two analyses should, when properly corrected, show the same figure for ear development. Simple correction figures, as shown below, were found to be sufficient. In all pairs of analyses, where the leaf numbers were between —.20 and —.80, the deviation between the two corrected stages of ear development was small. Due to unavoidable errors in leaf count and ear analysis, exactly the same figure could not be expected, and the deviation found could be explained as a result of such errors.

Correction in analyses where the leaf number was between —.30 and —.70. The

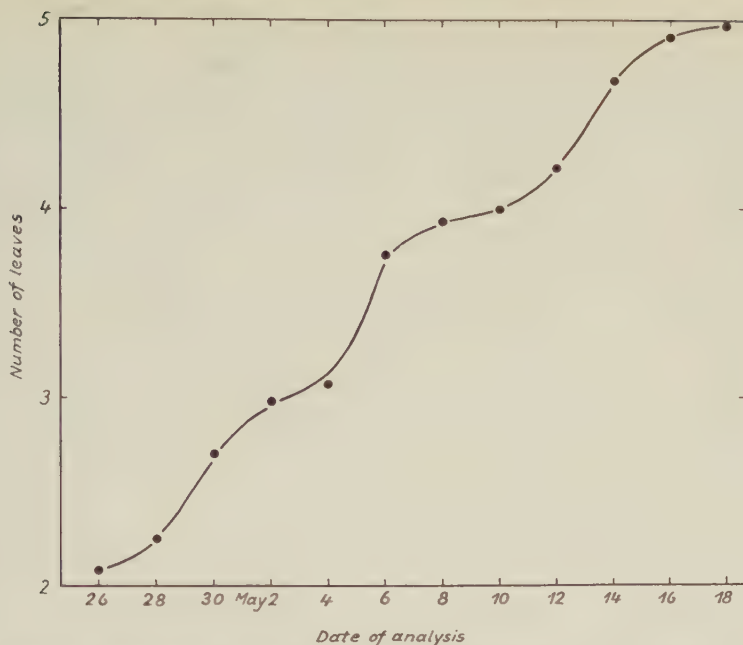


Figure 1. Number of leaves in a barley crop at successive dates. Counts from an experiment in 1952.

difference between the leaf number found and $-.50$ was calculated, and that figure was added to or subtracted from the figure for ear development. The largest correction in such analyses could be ± 0.20 .

Correction in analyses where the leaf number was between $-.20$ and $-.30$ or between $-.70$ and $-.80$. Due to the slower increase in leaf number at those stages (Figure 1), a more comprehensive correction was necessary. As above, the difference between the leaf number and $-.50$ was calculated, but to this correction figure was added the difference between the leaf number and $-.30$ or $-.70$ respectively. The largest correction in such samples could be ± 0.40 .

In a few cases it had been necessary to examine samples where the leaf number was between $-.80$ and $-.90$. Such analyses were corrected in the same way as analyses with a leaf number between $-.70$ and $-.80$. The largest correction was 0.60 . If the leaf number in a sample was below $-.20$, the sample was discarded and another collected the next day or two days later.

A couple of examples will show the procedure:

Example 1

Leaf number: 2.35; stage of ear: 1.87

Correction figure:

$$2.50 - 2.35 = 0.15$$

Corrected stage of ear: 2.02

Example 2

Leaf number: 2.76; stage of ear: 2.26

Correction figure:

$$2.50 - 2.76 = -0.26$$

$$2.70 - 2.76 = -0.06$$

$$-0.32$$

Corrected stage of ear: 1.94

Results

a. Effect of seeding rate and row distance. In one experiment with barley and oats, 5 different quantities of seed, varying from 50 to 800 kg per hectare were sown. In the same experiment the effect of row distance was tried, as each quantity, except 800 kg, was sown at two different row distances. However, variation of row distance was without effect. The figures in Table 1 show that changing the row distance has caused no change in leaf number, ear development, or in the percentage of plants with tillers.

The influence of different seeding rates appears in Table 2. The figures given show that ear development is more advanced after high than after low rate of seeding. In the case of oats the difference is only small, but in barley a large difference is found. The leaf number shows the opposite tendency. The smallest number of leaves was found in plots where 800 kg seed had been sown per hectare. With decreasing seed quantity sown, the leaf number increased and the highest number was found where 50 or 100 kg seed had been sown. A corresponding increase was found in number of plants with tillers. It should be mentioned that leaves from the tillers were not counted when figuring the number of leaves per plant. Here, as well in all other experiments, only leaves from the main shoot were counted.

The results are confirmed by two other experiments, where seeding rates from 25 to 200 kg per hectare were tried. In these experiments the number of leaves was highest where only 25 kg seed was sown. Ear development, on the contrary, was most advanced in plots with 200 kg seed.

When the growing points have reached stage 4 in barley and stage 3 in oats, their vegetative growth has ceased and they are transformed into ears. Using 50 kg seed per hectare, growth stage 4 in barley was reached when the leaf number was 3.77 (Table 2). Using 800 kg seed per hectare, the growth changed from vegetative to reproductive when the leaf number was considerably lower. It seems justifiable to assume that stage 4 was reached when the leaf number has been about 2.8. In oats a similar difference is found. The stage of the panicle was 3.5 and the leaf number 4.77 where 50 kg seed was

Table 1. *Effect of various row distance. Average figures from seeding rates varying from 50 to 400 kg per hectare.*

Distance between rows	Barley			Oats		
	Number of leaves	Stage of spike	Percent of plants with tillers	Number of leaves	Stage of panicle	Percent of plants with tillers
11 cm ...	3.65	4.4	43	4.54	3.8	29
22 cm ...	3.69	4.6	47	4.51	3.7	34

Table 2. *Effect of rate of seeding.* Field experiment sown on March 22, 1952. Analysis made on May 6 in barley, and on May 15 in oats.

Rate of seeding kg/ha	Number of leaves	Stage of spike or panicle	Percent of plants with tillers	Total number of leaves before heading
<i>Barley</i>				
50	3.77	4.0	59	8.46
100	3.84	4.0	70	8.40
200	3.60	4.8	39	7.76
400	3.50	5.1	11	7.75
800	3.27	5.2	0	7.18
<i>Oats</i>				
50	4.77	3.5	67	7.41
100	4.76	3.8	38	7.15
200	4.46	3.7	20	7.07
400	4.12	3.9	2	6.76
800	3.87	3.9	0	—

sown. In plots with a high seeding rate the stage of panicle is slightly higher, 3.9, but the leaf number is one unit lower.

The total leaf number was counted just before heading to ascertain whether this difference remained unchanged. This was found to be the case, and other experiments have shown the same (Table 7). These results seem to show that a fairly constant leaf number will appear after the close of the vegetative phase.

b. Effect of fertilizer treatments. Table 3 shows results from a field experiment in which the soil in some of the plots since 1899 has been subjected to exhaustion in plant nutrients. This experiment includes one treatment in which all the nutrients in question were applied, and 3 treatments in which a single nutrient has been omitted. The quantities of fertilizers applied annually to the fully treated plots correspond to 16 kg phosphorus and 45 kg potassium per hectare; nitrogen has been applied in quantities varying from 30 kg N to 100 kg N per hectare, according to the kind of crop grown. The more

Table 3. *Effect of fertilizer treatments.* Stage of development of the barley spike. Average of analyses from 1952 and 1953.

Treatment of plots from 1899 to 1953	Number of leaves			Percent of plants with tillers at the 3.5 leaf stage
	2.5	3.5	4.5	
Treated with K, P, and N	2.1	3.8	5.7	63
Without K	1.9	3.6	5.3	40
» P	2.0	3.9	5.5	36
» N	2.2	3.9	6.0	27

Table 4. *Effect of nitrogen.* Analyses from field experiment where calcium nitrate was applied immediately before or after seeding. Corrected stage of spike or panicle.

	Exp. I barley 1952	Exp. II barley 1952	Exp. III oats 1952	Exp. IV barley 1953		
Number of leaves	2.5	3.5	4.5	2.5	3.5	4.5
<i>Stage of spike or panicle</i>						
Without nitrogen	2.3	4.6	3.9	2.0	3.8	5.9
46.5 kg N per hectare...	2.0	4.3	3.7	1.8	3.5	5.4
<i>Percent of plants with tillers</i>						
Without nitrogen		38	25		63	
46.5 kg N per hectare...		54	39		87	

common crops were small grains and roots, and after 1928 no leguminous crops were grown. A 4-year rotation was used, and for that purpose the experiment was divided into 4 blocks, each with 3 replicates of the fertilizer treatment. Barley was grown in one of these blocks in 1952 and in another in 1953.

Another experiment was laid out on a soil of good fertility, but rather poor in available manganese. In this experiment potassium, manganese, and nitrogen were applied a few days before seeding. No effect of potassium and manganese could be shown, but a slight effect of nitrogen was observed (exp. IV in Table 4).

Table 4 summarizes some experiments in which nitrogen was applied just before or immediately after seeding of the crop. 300 kg of calcium nitrate per hectare was applied, corresponding to 46.5 kg nitrogen. In all experiments ear development was most advanced where no nitrogen was applied. In all cases the difference in ear development is small and not very important. However, as the difference is found in 4 independent experiments, an effect of nitrogen must be considered significant.

c. Variation in soil moisture. The experiments were made in wooden boxes, each containing 30—35 kg of soil. In each experiment there were 3 different treatments: a) no addition of water, b) application of sufficient water to keep the soil suitably moist, and c) excessive watering. In all treatments seed was sown in a uniform moist soil, and if necessary water was applied to all boxes in the time between sowing and emergence. In experiment number 3, due to a mistake, water was also applied just after emergence.

In experiment 1 seed was sown in a period of dependable weather so the boxes could be left out of doors. When the plants had developed 2.5 leaves, 7 mm rain fell unexpectedly. Another 7 mm of rain fell during the remaining experimental period. These small quantities of rain kept the plants alive in

Table 5. *Effect of variation in soil moisture. Stage of development of spike or panicle.*

Level of moisture	Exp. 1. Barley Number of leaves			Exp. 2. Barley Number of leaves			Exp. 3. Oats Number of leaves		
	2.5	3.5	4.5	2.5	3.5	4.5	2.5	4.5	5.5
Low	1.8	4.3	6.9	1.5	4.0	5.9	1.6	3.7	—
Medium	1.6	4.0	6.8	—	—	—	1.4	2.9	5.0
High	2.0	3.9	6.6	1.8	3.5	5.2	1.2	3.2	5.4

the boxes without watering, but much more was needed to keep the soil moist. In the artificially watered boxes water was applied in quantities corresponding to 40 and 80 mm precipitation.

Experiment 2 was made in a greenhouse during a period of high atmospheric humidity. Therefore the soil did not dry out to any great extent.

The boxes in experiment 3 were left out of doors when the weather seemed dependable, but were moved into a greenhouse when rain threatened. Here the soil in the boxes which were not watered became so dry that growth finally ceased almost completely. During one week the leaf number in the un-watered boxes increased only from 4.40 to 4.89, while in the watered boxes the increase was from 4.5 to 5.5.

In all three experiments, ear development was most advanced where plants had not been watered. The difference is not very great, but sufficient to indicate that soil moisture conditions are not without importance. In experiment 3 the plants with low water level wilted before reaching the leaf number 5.5.

d. Different times of seeding. In 1953 an experiment was made in barley with 10 different seeding times (Table 6). Due to a rainy period at the end of March, there was an interval between the second and the third seeding, but otherwise seedings followed in close succession.

The first 4–5 seedings show normal, or slightly above normal, figures for ear development. The figures for plants sown later are lower, particularly for those sown on April 23, April 27, and April 30.

The figures might indicate a correlation between seeding date and ear development. However, as other experiments did not show a similar correlation, that theory could not be correct. It seems more reasonable to assume that the low figures for spike development were due to a period of cold weather. The period from May 9 to May 15 was rather cold and cloudy. Official meteorological reports show that the average temperature in the week, May 10–May 16, was 4° C lower than in the previous week. In reality, in the field it was still lower, but on May 15 the weather changed suddenly, and the temperature, May 16, was very high. It seems reasonable to assume

Table 6. *Effect of various time of seeding. Stage of development of the barley spike in field experiment 1953.*

Date of seeding	Date of emergence	Number of leaves		
		2.5	3.5	4.5
March 18	April 6	2.7	4.7	7.3
» 25	» 13	2.1	—	6.9
April 10	» 23	1.9	3.8	6.0
» 13	» 27	1.6	4.0	6.3
» 18	» 30	1.7	4.4	5.5
» 23	May 3	—	3.4	5.1
» 27	» 6	2.1	3.5	4.7
» 30	» 10	1.9	3.2	5.2
May 4	» 16	1.7	—	6.3
» 7	» 18	2.1	4.0	6.6

that ear development was retarded in those plants where transformation from vegetative to reproductive growth occurred in the cold period. It was impossible to prove this assumption by experiments made under controlled temperature and light conditions.

e. Varieties, stocks of seed, and localities. In 1952—53 a rather large number of varieties, mainly Scandinavian, were studied. Considerable varietal differences were found (Andersen 2).

Figures from those trials show that differences found at an early stage of development will last until heading takes place (Table 7). In 6 varieties of barley, plants were examined at 5 different stages, the last when the plants had developed 6.5 leaves. Furthermore, in 1952 the total number of leaves was counted just before heading. Among the 6 varieties, 2 were early, 2 medium, and 2 late in ear development. In the early varieties, stage 4 must have been reached when the leaf number was about 3.9 and in the late varieties at 3.5. From this time the difference between early and late varieties remains constant, the total number of leaves being greatest in the late varieties.

By courtesy of the Danish Seed Testing Station, the author was allowed to draw samples from 25 plots with Rigel barley in 1953. Each plot represented a certain stock of seed and the different stocks were presumably grown on different fields. The samples were analysed when the leaf number was 3.5, and small differences in ear development were found. Therefore the investigation was repeated in 1954, using the same stocks of seed. The average figures found for the two years indicated no difference between the different stocks.

Nearly all the analyses mentioned here were made from experiments at the experimental station of the Agricultural College. In order to study development in plants from other parts of Denmark, analyses were made

Table 7. *Stage of development of the spike in two early (e), two medium (m) and two late (l) varieties of barley. Average figures from field experiments 1952 and 1953.*

Variety	Number of leaves					Total number of leaves 1952
	2.5	3.5	4.5	5.5	6.5	
Rex II (e)	2.7	5.2	7.5	9.0	10.3	7.35
Freja (e)	2.2	5.1	7.8	8.9	10.1	7.83
Rigel (m)	1.9	4.5	6.9	7.9	9.8	7.78
Maja (m)	2.3	4.4	7.0	8.2	9.7	7.93
Carlsberg (l)	1.8	4.0	6.1	7.2	9.0	8.72
Fero (l)	1.6	4.0	5.8	7.0	9.1	8.48

in 1952 and 1953 on plants collected at various places and on various soil types. Some differences in development was observed from one place to another, but not greater than might well be due to difference in variety, time of seeding, etc.

f. Experiments with day-length and light intensity. In 1953 flower-pot experiments were made in order to study the effect of short-day treatment on the development of barley plants. In a preliminary experiment the effect of various day-length was studied, and in the main experiment plants received short-day or long-day treatment at different stages of growth. Due to lack of facilities the experiments were started rather late in the season, but the results found will presumably be applicable to barley sown at normal time.

The preliminary experiment showed a slight depression of the spike in plants grown in a 12-hour day, but no differences were found between plants grown in a 14-hour day and plants grown in natural day-length (17 hours). If the daily exposure to light was less than 10 hours, barley did not head at all. Based upon those observations, the length of a »short day» in the main experiment was fixed at 9 hours. The normal day-length at this time was 17—18 hours (»long day»).

The main experiment fell in two series. In one of these, plants received short-day treatment at different times. The plants were grown in natural day-length (long days), but in periods of 4 days a number of pots were covered after 9 hours daily exposure to light. Four such periods of 4 days length were used, the first one began at the day of emergence, May 25; the last one, 12 days later (Table 8). The plants received short-day treatment in one 4-days period only, or in two succeeding periods.

In the second series the effect of long-day treatment was studied. The plants in this series were grown in a 9-hour day, but exposed to long-day treatment in periods of 4 or 8 days. In this series, the plants were covered with boxes of a less heavy construction than in the first series and in the preliminary

Table 8. *Effect of long- or short-day treatment at different stages of development. Pot experiment with barley 1953. Analysis made on June 10.*

Time of treatment	Short-day treatment Control plants grown in long days			Long-day treatment Control plants grown in short days		
	Number of leaves	Stage of spike	Percent of plants with tillers	Number of leaves	Stage of spike	Percent of plants with tillers
Control	3.88	4.9	35	3.83	4.0	7
4-days treatment						
May 25 to 29	3.71	5.1	2	3.83	4.0	11
» 29 to June 2	3.34	4.7	0	3.65	4.1	4
June 2 to 6	3.66	4.9	7	3.87	4.0	47
» 6 to 10	4.00	4.9	29	3.79	3.9	6
8-days treatment						
May 25 to June 2	3.80	4.4	35	3.57	4.3	7
» 29 to » 6	4.00	3.4	81	3.47	5.0	2
June 2 to 10	3.72	4.4	11	3.73	4.4	27

experiment. Such covering was not sufficient to prevent spikelet formation, but the control plants reached a development stage of 4.0.

It is seen from Table 8 that a 4-day treatment was without effect, whether the days were long or short. The 8-day treatment, May 29—June 6, however, has influenced development of the spike. The stage of spike was low in plants receiving short-day treatment in those days, and high in plants exposed to long-day treatment.

Experiments on the influence of light intensity were made using a very simple method. A number of plants in flower-pots were left outdoors, while others were covered with a hot-bed frame in which two thirds of the glass area was covered with wooden lists. The ends of the hot-bed frame had been removed to permit air circulation and thus prevent a rise in temperature. As in the day-length experiment, the plants were shifted and subjected to treatment during periods of 5 and 10 days.

Plants kept in shadow during the whole experimental period showed, on May 22, a lower leaf number, but a higher stage of spike, than plants left in the open showed on May 15 (Table 9). Similar results were observed in other experiments. Reduction in light intensity always reduced vegetative growth more than ear development. If plants with the same number of leaves are compared, the ear will have reached a more advanced stage in shaded than in unshaded plants.

The results found were practically independent upon time of treatment. The figures in Table 9, therefore, are averages from 3 experimental periods, each lasting 10 days. The first of these began immediately after emergence; the last one ended on May 15.

Table 9. *Effect of light intensity. Pot experiment with barley 1954.*

Treatment	Analysed on May 15			Analysed on May 22		
	Number of leaves	Stage of spike	Percent of plants with tillers	Number of leaves	Stage of spike	Percent of plants with tillers
Open air all time...	3.66	5.1	68	4.70	6.9	81
Open air, except 10 days in shadow	3.12	4.0	22	4.21	6.4	55
Shadow, except 10 days in open air	3.06	4.1	14	3.92	5.9	25
Shadow all time ...	2.90	3.9	2	3.30	5.7	0

Discussion

In discussing the possible error introduced by leaf counting, it is taken for granted that ear development is fully applicable as a method to characterize development of the entire plant. Leaf count is a less accurate, but quicker method, and much time could be saved by using it. Leaf count can be made in the field, eventually on growing plants, whereas determining the stage of ear development belongs in the laboratory.

If plants are grown under conditions deviating greatly from normal (i.e. short day, winter culture, culture in greenhouses etc.) leaf counting will be insufficient to characterize their stage of development. The same is true if extremely small or extremely large quantities of seed are used. Normally, the seed quantity sown will be fairly constant, but if many plants are injured by disease or insect pests, conditions similar to lower seed rating may arise. In such crops, ear development on the main shoots will be somewhat retarded and many tillers will appear which lag behind the main shoot in development.

Among the factors investigated, soil fertility and soil moisture did not greatly affect the relation between leaf number and ear development, and may be neglected in statistical treatment of results from a series of experiments. If a number of different varieties are investigated, large differences are found. Due attention must be paid to such differences when leaf count in different varieties are compared.

Climatic factors are evidently very important. This investigation and Mitchell's experiments with rye grass (8) indicate that the degree of tillering depends on the relation between temperature and light intensity. Since the correlation between ear development and number of tillers is negative, an advanced stage of ear development could be expected when the number of tillers is low. If leaf count is made late, attention could be paid to the degree of tillering, but if leaf count is made in a young crop, climatic conditions

Table 10. *Extreme high or low stages of ear development found in 5 years experiments with Rigel barley and Stål oats. Two crops from each species in which ear development was initiated when leaf number was very low or very high. Field experiments with a seeding rate of 180 to 200 kg per hectare.*

Year and date of seeding	Number of leaves				
	2.5	3.5	4.5	5.5	6.5
<i>Abed Rigel barley</i>					
March 31, 1949 ...	3.5	5.3	7.2		
April 30, 1953 ...	1.9	3.2	5.2		
<i>Svaløf Stål oats</i>					
March 21, 1952 ...		1.7	4.4	6.1	7.4
March 18, 1953 ...		1.0	2.5	4.4	6.2

must be considered as a source of error in applying the method. This would also be the case, had it been possible to make experiments in greenhouses under controlled conditions. Such experiments, therefore, would be of no value in practical application of the leaf counting method, but would, of course, be of considerable theoretical interest.

Since the increase in leaf number is discontinuous, but the ear develops continuously, the correlation between leaf number and ear development depends on the size of the leaf number. The closest correlation is found when the average leaf number is half-way between two whole figures. When the leaf number approaches a whole figure, the very slow increase in leaf number is a source of error.

For a certain leaf number the development stage of the ear must lie between an upper and a lower limit. From a practical point of view, the most extreme values caused by short days or other unusual treatments are not essential. More important are the limits found in plants grown under normal field conditions. Such limits appear in Table 10. In 1949 spike development in barley was initiated when the leaf number was low; in 1953 when the leaf number was one unit higher. In oats, the panicle started growth early in 1952, but late in 1953. The figures are from field experiments on a soil in good fertility. 180—200 kg seed were sown per hectare. Under these conditions, figures, below or above those shown, were very rare in Rigel barley and Stål oats.

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Summary

In spring-sown barley and oats the relation between leaf number and ear development was investigated in a series of analyses. In the case of one single variety this relation was affected by weather conditions and by seeding rate, but the effect from soil fertility and soil moisture was inappreciable. There was a strong negative correlation between development of tillers and ear development in the main shoot.

When using leaf count instead of ear development for growth analyses in spring-sown oats and barley, three sources of error exist: 1) error due to the influence of weather conditions and other uncontrollable factors (Table 10), 2) errors due to the fluctuating increase in average leaf number (Figure 1), and 3) errors due to varietal differences and different seeding rates. Where a high degree of accuracy is required, examination of the ear could not be replaced by leaf count. Where less accuracy is necessary, counting of the leaves is an easy and useful method.

References

1. Andersen, S.: Methods for determining stages of development in barley and oats. — *Physiol. Plant.* 5: 199. 1952.
2. — Skadevirkning på korn ved sprøjtning med 2,4-D og 4K—2M. — *Tidsk. f. Planteavl* 1955.
3. Borthwick, H. A., Parker, M. W. & Heinze, P. H.: Effect of photoperiod and temperature on development of barley. — *Bot. Gaz.* 103: 326. 1941.
4. Gardner, J. L.: Studies in tillering. — *Ecology* 23: 162. 1942.
5. Gustafson, F. G.: Influence of external and internal factors on growth hormones in green plants. — *Plant Physiol.* 21: 49. 1946.
6. Hamilton, H. H.: Developmental study of the apical meristem in four varieties of *Avena sativa* grown at two temperatures. — *Amer. J. Bot.* 35: 656. 1948.
7. Leopold, A. C.: The control of tillering in grasses by auxin. — *Amer. J. Bot.* 36: 437. 1949.
8. Mitchell, K. J.: Influence of light and temperature on the growth of ryegrass (*Lolium* spp.). II. The control of lateral bud development. — *Physiol. Plant.* 6: 425. 1953.

Studies on the Balance between Free and Bound Auxin in Germinating Maize

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The investigations by Haagen-Smit, Leech and Bergen (1941) and by Berger and Avery (1944) have shown that the acid auxin of maize kernels is indoleacetic acid. A large part of this auxin occurs as a precursor which, according to Avery, Berger and Shalucha (1942 a), can be transformed into auxin by alkaline hydrolysis.

According to Hatcher and Gregory (1941), one cannot demonstrate the presence of auxin in the ovules of rye until two weeks after anthesis. The auxin content then rises to a maximum which is reached in five weeks following anthesis, after which it again falls to a minimum as the caryopses lose water. Hatcher (1943) shows that the lowering of the auxin level is accompanied by the formation of an inactive auxin precursor from which auxin can be obtained by alkaline hydrolysis.

Avery, Berger and Shalucha (1942 b) show that the auxin content is low in the ovules of maize during the time preceding pollination but that it starts rising immediately after, reaching a maximum in 1—3 weeks after pollination. The amount of auxin then decreases anew. The bulk of the auxin occurs as precursor. Avery et al. were unable to demonstrate the existence of any fixed relationship between the amount of auxin precursor and the amount of free auxin.

Hinsvark, Houff, Wittwer and Sell (1954) demonstrated the presence of small amounts of indoleacetic acid in very unripe maize kernels by means of colorimetric methods. As ripening proceeds, the amount is increased to a

maximum, which is reached while the kernels are still unripe. Afterwards the indoleacetic acid content falls off again, so that no indoleacetic acid can be demonstrated by their methods in fully ripened maize kernels. During the stage at which the indoleacetic acid content is at its maximum, Hinsvark et al. also found ethyl-indoleacetate in the kernels.

It has thus been demonstrated that the auxin in seeds is transformed during their ripening process into inactive precursors, with the result that the auxin content in ripe seeds is comparatively low. Only a part of the auxin extracted with water or ether from the seeds studied by the above-mentioned authors, is likely to have been free auxin. The greater part of it was presumably bound in one way or another and was only liberated during the course of the extraction, so-called bound auxin. In the present paper an account will be given of some experiments showing the balance between such free and bound auxin in maize kernels at different stages of germination, and the influence of externally administered indoleacetic acid on this balance. A preliminary report of some of the results was given in a previous publication (Hemberg, 1954 c).

The Natural Auxin Balance in Dormant and in Germinating Maize Kernels

Linser (1939) has shown that the auxin content of barley caryopses increases during swelling. In the course of subsequent germination the auxin content falls off slowly. Funke (1943) is of the opinion that the temporary increase of auxin activity in Linser's experiments was caused by the disappearance of inhibitory substances rather than by a real increase in auxin content.

Avery, Creighton and Shalucha (1940) applied different extraction methods to dormant and germinating maize kernels. When they extracted the kernels with water, they found the highest auxin activity in extracts of dry kernels. The auxin activity became successively lower in extracts prepared from kernels in a more and more advanced state of germination. When they used ether or alcohol or a mixture of both as extraction solvents, they obtained much lower auxin activities; and the maximum auxin activity was found in extracts of kernels that had been swelling for 24 hours, whereas longer periods of swelling reduced the level of auxin activity.

von Guttenberg and Lehle-Joerges (1947) have investigated the presence of auxin i.a. in aqueous extracts of maize kernels. They find, in contrast to the results obtained by Avery and his co-workers with the same extraction method, that the content of auxin increases in the kernels during the first 24 hours of swelling, after which it again decreases.

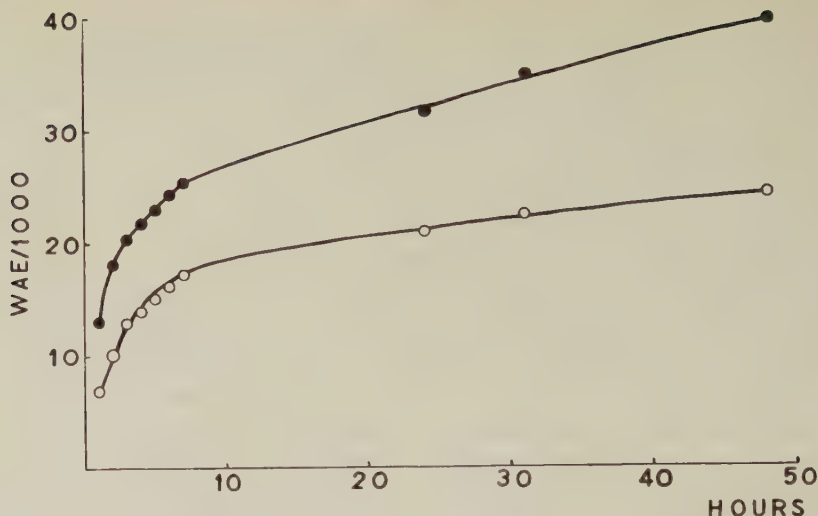


Figure 1. Extraction of maize kernels after 48 hours of swelling at $+28^{\circ}\text{C}$. Temperature during extraction: $+2^{\circ}\text{C}$ (○) and $+28^{\circ}\text{C}$ (●). Abscissa: extraction time in hours. Ordinate: total amount of extracted acid auxin, expressed as WAE/1000 per maize kernel.

Sircar and Das (1951) find that in rice the auxin content of the endosperm decreases during the course of germination.

Yamaki and Nakamura (1952) have studied in a comprehensive investigation the auxin content and the tryptophane content of maize kernels at different stages of germination. They distinguish between free auxin, both neutral and acid, and bound auxin. But they do not use these terms in the customary sense (van Overbeek, de Vazquez and Gordon, 1947). Owing to the treatment of the material and the length of the extraction period, the greater part of what they call free auxin must consist of auxin liberated from bound stages in the course of extraction.

Method. — Sugar maize of the »Express» variety from W. Weibull Ltd. of Landskrona was used in the experiments. The kernels were extracted with wet peroxide-free ether. Before extraction, dry kernels were ground in a mill, while swelled or germinated kernels were ground with mortar and pestle. All the kernels examined, whether swelled or germinated, were soaked for 10 minutes before swelling in a 1 per cent calcium hypochlorite solution for the prevention of fungus growth. After thorough rinsing they were left in water at $+28^{\circ}\text{C}$ for a further 2 hours and 50 minutes. Thus they lay in water for 3 hours altogether. Some of the swelled kernels were extracted immediately after swelling; others were placed on wet filter paper in large petri dishes at $+28^{\circ}\text{C}$, and samples were extracted after various periods of time.

In the extraction the same distinction was made between free and bound auxin as in earlier experiments (Hemberg, 1951, 1954 a and b). The auxin extracted during

Table 1. Amount of acid auxin, expressed as WAE/1000 per kernel, extracted at different temperatures from maize kernels that had swelled for 24 hours. The first extraction lasted for 3 hours with a change of ether once per hour. The second extraction lasted for 45 hours during which time the ether was changed twice.

First extraction			Second extraction			Total amounts from first and second extractions
Temp.	Amount of auxin		Temp.	Amount of auxin		
	Det.	Average		Det.	Average	
+ 2° C	27.2 27.5	27.4	+ 28° C	37.6 38.0	37.8	65.2
+ 28° C	32.0 36.0 37.0	35.0	+ 28° C	34.0 34.5 36.0	34.8	69.8

the first 3 hours was called free auxin. This extraction took place at +2° C, the ether being changed every hour. The auxin extracted during the following 45 hours was called bound auxin. The second extraction took place in an incubator at +28° C, the ether being changed twice. Figure 1 gives an idea of the course of the extraction at the two temperatures. At both temperatures most is extracted during the first hour, after which the yield falls off very considerably. The yield is higher at +28° C than at +2° C; this is evident already during the first hour. It is thus plain that the liberation of auxin from bound precursors proceeds more easily in the warmth than in the cold, and that it proceeds so rapidly that, in the extraction of free auxin, it is not sufficient to make the extraction during a short period, but it must also take place in the cold. See also Table 1.

The extracts were fractionated according to Boysen Jensen's method II (1941), and the acid fraction alone was assayed for auxin. The auxin content of the extracts was determined by means of the Avena test in the form previously used (Hemberg, 1947).

The auxin values obtained are expressed in WAE units, as defined by Boysen Jensen (1941). Nowadays the auxin values are usually expressed in μg indoleacetic acid. To do this, the sensitivity of the Avena test to synthetic indoleacetic acid is examined every day. In his own experiments, however, the present author has found this to be a rather unsuitable method, as the activity decreases in crystalline indoleacetic acid after lengthy storage. If one has several preparations of different age, these can thus exhibit a varying degree of activity. As the test plants used in the cultivation method recommended by Boysen Jensen (1937) appear to retain approximately the same sensitivity throughout the entire year (Juel, 1941, Hemberg, 1947), it would therefore seem more appropriate to express the auxin values in the relative unit WAE.

Results and Discussion. The Auxin Content in Dormant Maize Kernels. — The content in dry intact maize kernels was determined on 3 occasions. The results (see Figure 2 A) show that the kernels, when extracted on May 19, 1954, contained large amounts of bound auxin, while they contained far less at the extraction on August 20 and still less on September 1. The experiments were made with kernels from the same harvest; these had been kept in the laboratory in the intervals between the extractions. Consequently, the diffe-

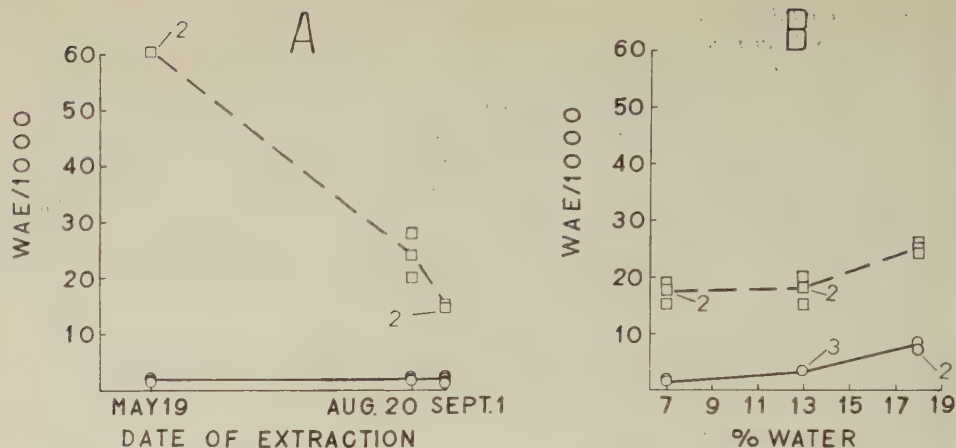


Figure 2. Amount of free (O) and bound (□) acid auxin in dry maize kernels. A. The kernels extracted at different times in 1954. Abseissa: date of extraction. Ordinate: as in Fig. 1. B. Kernels with different water contents extracted on Oct. 27, 1954. Abseissa: water content as percentages. Ordinate: as in Figure 1.

rences must be due to changes occurring in the kernels as a result of storage. Among other things, the kernels had lost water. There is a possibility that it might be easier to extract bound auxin from comparatively wet maize kernels than from comparatively dry ones.

For a closer study of the significance of the water content in relation to the auxin yield during extraction, kernels with different water contents were extracted with ether. The experiment was made on October 27, 1954, with kernels from the same harvest (1953) as those used in the preceding experimental series. They had been kept for 9 days in desiccators over silica gel, saturated sodium chloride solution or distilled water. The resulting water contents of the kernels were 7.1, 12.9 and 18.1 per cent respectively. At that time untreated kernels contained about 10 per cent.

The experiment shows (see Figure 2 B) that the content of bound auxin is equally great in kernels with a 7.1 per cent water content and in kernels with a 12.9 per cent water content. This difference in the water content of the kernels is thus of no significance for the result of the extraction. The content of free auxin, also, is roughly the same in the two lots of kernels. The kernels containing 18.1 per cent of water were soft and had begun to swell slightly. In connection with this incipient swelling their amounts of bound as well as of free auxin had slightly increased. The content of bound auxin in extracts from these kernels was, however, still much lower than it was in extracts prepared from dry kernels on May 19 (see Figure 2 A and B).

It is clear from the experiment that within certain limits the water content of the kernels is of no particular significance for the yield of bound auxin during the extraction. The differences in content of bound auxin demonstrated in extracts prepared on different occasions from dry maize kernels, cannot therefore be explained as a result of decreasing water content in the kernels. They must instead be due to the fact that the auxin precursors which yield »bound auxin» were present in greater amounts at the extraction on May 19 than at the later extraction dates. While the maize was being stored, there evidently took place a gradual transfer of auxin precursors with comparatively easily extractable auxin to auxin precursors with more firmly bound auxin. This seems to be a continuation of the process that Hatcher (1943) found to take place in ripening rye caryopses. Juel (1941) also found that the auxin content of the seeds decreases during storage. The auxin amounts determined by her comprise free as well as bound auxin, as she made use of ether extraction over a long period, 2 days, at room temperature.

The fall in the yield of bound auxin (Figure 2 A) might also be explained by assuming that the enzymatic activity had decreased during storage. However, this should not merely affect extraction of the bound auxin but also extraction of the free. In kernels with a low enzymatic activity one would expect to find a smaller amount of free auxin than in kernels with a higher enzymatic activity. However, in all the extractions the same amount of free auxin was demonstrated in the kernels (see Figure 2 A).

The Auxin Content of Maize Kernels at Different Stages of Swelling. — In four experimental series maize caryopses at varying stages of germination were extracted. In the first series the extractions began on May 19, 1954. It comprised dry kernels and kernels that had swelled for 3, 24, 48 and 96 hours. In the last two lots of kernels both roots and coleoptiles had formed. As already mentioned (see p. 421), the dry maize kernels in this series contained large amounts of bound auxin. In the extract from kernels that had swelled during 3 hours the content of bound auxin was lower (see Figure 3 A), and it was still lower in the extracts prepared from kernels that had swelled 24 and 48 hours respectively. Concurrently with the fall in the amount of bound auxin there was a rise in the amount of free auxin, so that this, which was very low in the extract of dry kernels, was higher in the extract prepared from kernels that had swelled for 3 hours and still higher in extracts of kernels that had swelled for 24 and 48 hours respectively.

The extracts in the next experimental series (see Figure 3 B) were prepared on August 20, 1954. As already mentioned, the content of bound auxin in the dry kernels was here much lower than in the previous series. In the extract of kernels that had swelled during 3 hours the content was much higher than in the extract of dry kernels, but it was again lower, just as in the previous

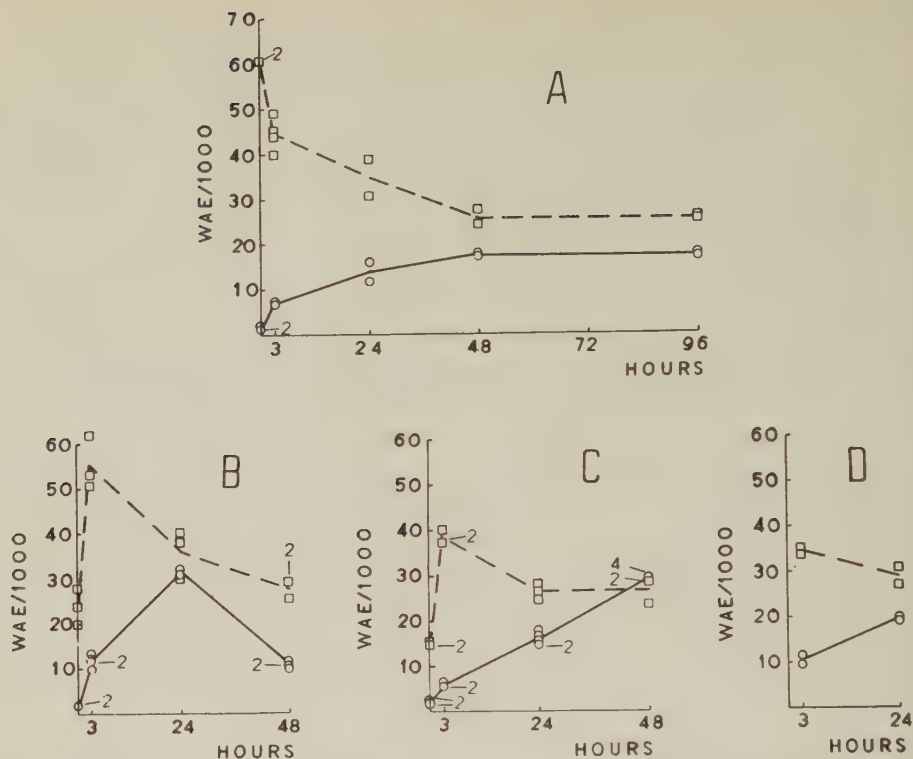


Figure 3. Amount of free (○) and bound (□) acid auxin in maize kernels at different stages of swelling. Abscissa: swelling time in hours. Ordinate: as in Fig. 1. The extractions began on May 19 (A), Aug. 20 (B), Sept. 1 (C) and Nov. 16 (D), 1954.

series, in the extracts prepared after 24 and 48 hours' swelling respectively. The content of free auxin was low in the extract prepared from dry kernels and higher in the extract of kernels that had swelled during 3 hours. The largest amount of free auxin was present in the extract from kernels that had swelled for 24 hours, while the extract of kernels that had swelled for 48 hours — in contrast to the preceding series — again contained a smaller amount. This might, however, be due to losses caused by fractionating the extract.

The results of the third experimental series (see Figure 3C) coincide in the main with those of the preceding series. The amount of bound auxin is low in the extract of dry kernels and much higher in the extract of kernels that had swelled for 3 hours; and it is again lower in the extract of kernels that had swelled for 24 hours. The amount of free auxin is lowest in the extract of dry kernels and rises successively in the extracts of kernels swelled for 3, 24 and 48 hours respectively.

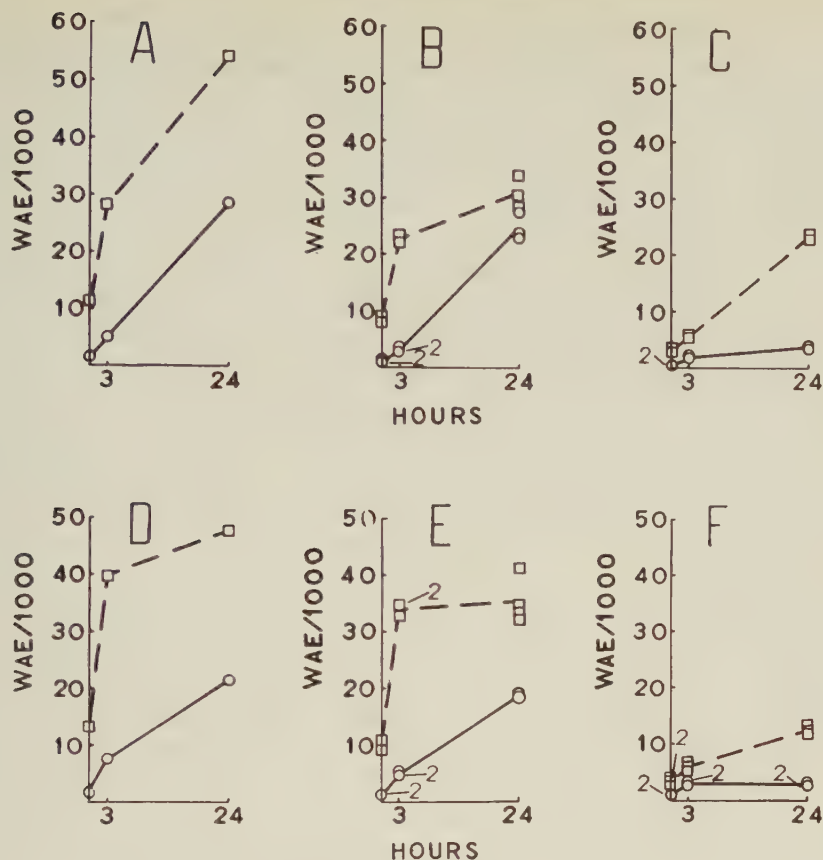


Figure 4. Amount of free (○) and bound (□) acid auxin in maize kernels at different stages of swelling. Endosperm and embryo were extracted separately. A—C. The extractions begun on June 16; D—F. The extractions begun on Nov. 23, 1954. A and D. Amounts of auxin from the endosperm and embryo extracts taken together. B and E. The auxin amounts in the endosperm extracts. C and F. The auxin amounts in the embryo extracts. Abscissa and ordinate: as in Figure 3.

In the fourth experimental series, finally, which only comprises kernels swelled for 3 and 24 hours respectively (see Figure 3 D), the results agree well with the results obtained in the three previous series.

In two experimental series the kernels were allowed to swell as before, but embryo and endosperm were extracted separately. If we add together the amounts of bound auxin in extracts of endosperm and in extracts of embryos from different swelling stages, we find that extracts of dry kernels or of kernels swelled during 3 hours no longer contain the greatest amount of bound auxin, as they did in the preceding series in which endosperm and embryo

were extracted together. Instead, extracts of kernels that have swelled for 24 hours contain most bound auxin (see Figure 4 A and D). If we consider the extracts of endosperm and embryo separately, we find, in the case of the endosperm extracts (see Figure 4 B and E), that in extracts of dry kernels there is little bound auxin and in extracts of kernels swelled for 3 hours considerably more. In extracts of kernels that have swelled for 24 hours, there is, in one series at least, just as much as in the immediately preceding extract, and in another series more. The largest amount of free auxin is present in extracts of kernels swelled for 24 hours and the smallest amount in extracts of dry kernels. If we consider the embryo extracts (see Figure 4 C and F) we find that extracts of dry kernels and of those that have swelled for 3 hours contain relatively little bound auxin, while extracts of kernels swelled for 24 hours contain much more. There is very little free auxin present in the embryo extracts. In one of the experimental series the largest amount occurs in the extract of kernels swelled for 3 hours, while in the other the extract of kernels swelled for 24 hours contains slightly more than the extract of those swelled for 3 hours.

In extracting intact maize kernels, we are thus able to establish an increase in the amount of bound auxin, i.e. of auxin precursors, during the first hours of swelling, if the content of the kernels in a dry state is low, but a decrease, if the content of bound auxin in the dry kernels is high. Subsequently, there takes place in all the series during the next 24 hours of swelling a reduction in the amount of bound auxin. Concurrently with this reduction of the bound auxin, there is an increase in the amount of free auxin. This must be derived from the bound auxin, i.e. from auxin precursors.

If we consider the experiments on intact maize caryopses, the data obtained in the series of May 19, 1954, indicate that the total content of auxin (the sum of free and bound auxin) decreases slowly during the first 48 hours of germination. This result is in agreement with those of Avery, Creighton and Shalucha (1940). The data obtained in the series of August 20 and September 9 indicate that the total auxin content first rises steeply during swelling and then falls off somewhat. Similar findings were reported by von Guttenberg and Lehle-Joerges (1947). In the series of Sept. 9 (Figure 3 C) extracts of kernels swelled for 24 and 48 hours respectively, admittedly contain equal amounts of bound auxin, but in this series, too, the extract prepared from kernels that have swelled for 24 hours is lower in bound auxin than the extract prepared from kernels swelled for 3 hours.

The fact that the result of the extraction is entirely different when endosperm and embryo are extracted separately, may probably be explained as follows. Auxin precursors are likely to be present most abundantly in the endosperm, while the bulk of the enzymes which can form auxin from these

precursors during ether extraction, are present in the embryo. In a prolonged ether extraction of intact kernels that have swelled for 3 hours, a large amount of the endosperm's auxin precursors can consequently, during the extraction, be transformed into auxin, by the action of the embryo's enzymes. If, on the other hand, endosperm and embryo are extracted separately, a large amount of auxin precursors is certainly present in the endosperm, but there is insufficient enzyme to transform any considerable quantity of these into auxin during a prolonged extraction. Accordingly, smaller amounts of bound auxin are obtained in extracting isolated endosperm tissue from maize kernels that have swelled for 3 hours than in extracting entire maize kernels of the same degree of swelling. In the embryo, there is at this time no substantial amount of auxin precursors. If, however, the kernels are allowed to swell for 24 hours, part of the precursor material which was initially present in the endosperm, will be transferred to the embryo. If the embryo is isolated after 24 hours of swelling, therefore, it does contain a certain amount of precursor, which can serve as a substrate for the conversion enzymes. Consequently, auxin should be released during prolonged ether extraction.

Yamaki and Nakamura's (1952) results are difficult to compare with those described in the present paper. This is partly due to differences in extraction technique. As they extracted their material by comparatively violent methods, this is likely to have affected the yield of auxin. The fact that they found only small amounts of »free auxin» in the endosperm and much more in the embryo, is noteworthy.

The Fate of Externally Administered Indoleacetic Acid in Germinating Maize Kernels

Many attempts have been made to influence the germination of seeds by addition of indoleacetic acid. The reader is referred to Kruyt (1954) for a review of this subject.

Siegel and Galston (1953) have shown that if indoleacetic acid is administered to excised pea roots it will be bound to proteins in the roots. Also in vitro the proteins of pea roots are capable of coupling with indoleacetic acid.

Dettweiler (1942) found that the auxin content in the *Coleus* internode rises if the internode is coated with indoleacetic acid paste. He claims that the increase in auxin activity is due to neoformation of auxin *a*, provoked by the action of indoleacetic acid. He believes that he can identify auxin *a* by its stability to boiling with HCl. However, investigations by Bentley and Housley (1953) have shown that indole acetonitrile, which occurs naturally in plants, is stable to boiling with acids. It seems reasonable, therefore, to assume that

the applied indoleacetic acid has been converted to indole acetonitrile in the plant. The possibility exists that such conversion might be one step in the transformation of free into bound auxin. To test the correctness of the hypothesis that externally applied indoleacetic acid can form bound auxin in the plant, some experiments have been made in which the content of free and of bound acid auxin was determined in untreated maize caryopses and in caryopses treated with indoleacetic acid, the kernels in both cases being at different stages of swelling.

Of 6 lots of dry maize kernels, with 50 kernels in each lot, 2 lots were placed in distilled water, 2 in indoleacetic acid solution, 10 mg per litre, and 2 in indoleacetic acid solution, 100 mg per litre. After 3 hours at $+28^{\circ}\text{C}$, the kernels were well rinsed and carefully dried with filter paper. One lot of kernels from each solution was immediately subjected to ether extraction, while the other was placed on wet filter paper in a large petri dish and allowed to swell for a further 21 hours at $+28^{\circ}\text{C}$. Afterwards this lot was also extracted. In both cases free and bound acid auxin were distinguished as previously described.

The results show that kernels which were treated for 3 hours with indoleacetic acid solution contained more free acid auxin than those soaked in pure water (see Figure 5 A). The extract of kernels from the weaker solution contained twice as much and the extract of kernels from the stronger solution 30 times as much as the extract of kernels soaked in pure water. The amount of bound auxin also increased in kernels treated with indoleacetic acid but far less than the amount of free auxin. Thus the extract of kernels from the weaker solution contained 1.5 times as much, and the extract of the kernels from the stronger solution 5 times as much as the extract of those soaked in water.

The situation was different in the case of kernels that had lain for a further 21 hours on wet filter paper. In these the content of free auxin was certainly higher in the lots that had lain in indoleacetic acid solutions than in the lot that had lain in water, but the differences were no longer so great. The extract of kernels from the strongest indoleacetic acid solution now contained only about 5 times as much free auxin as the extract of those that had lain in water. The extract of the kernels from the weakest solution still contained twice as much as that of the water-treated ones. But now one found great changes in the content of bound auxin in those kernels that had been treated with the stronger indoleacetic acid solution. The extract of these kernels contained at this time 12 times as much bound auxin as the corresponding extract of the water-treated ones, while the extract of kernels from the weaker solution merely contained twice as much as the extract of the water-treated ones (see Figure 5 B). Evidently, the strongest indoleacetic acid solution has induced the formation of considerable quantities of bound auxin in

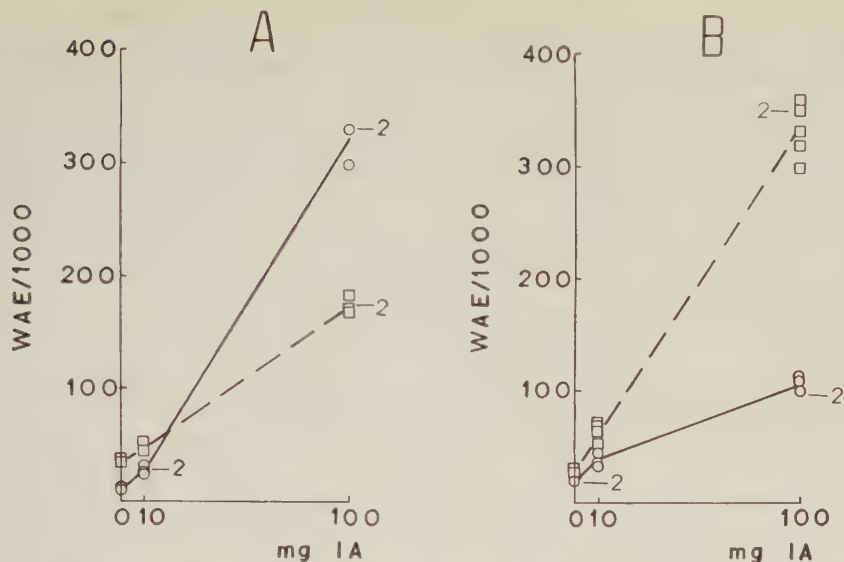


Figure 5. Amount of free (○) and bound (□) acid auxin in maize kernels that had swelled for 3 hours in pure water or in indoleacetic acid solutions, 10 and 100 mg per litre respectively. A. Immediately after swelling. B. After the kernels had been allowed to swell for a further 21 hours on wet filter paper at +28° C. Abscissa: mg indoleacetic acid per litre of the solution in which the kernels were soaked. Ordinate: as in Figure 1.

24 hours. It seems, therefore, that the enzymes which transform bound auxin to free, can also transform free auxin to bound. The direction which the reaction is to take is determined by the substances present. If a large surplus of free auxin is present, the reaction, according to the law of mass action, must thus take the direction from free to bound auxin.

Hitherto, the chemical nature of the bound auxin has not been investigated. It is, however, reasonable to assume that tryptophane may in some way play a part in the formation of this auxin. According to Stehse and Wildman (1950) tryptophane is enzymatically converted to indoleacetic acid in maize kernels. The auxin thus formed, subsequently enters an »auxin complex», which may be regarded as a potential source of auxin, an auxin precursor. It is also quite possible that tryptophane itself constitutes an auxin precursor (for literature, see Gordon, 1954).

The capacity of maize kernels rapidly to convert tryptophane to acid auxin was demonstrated in the following experiment. Six lots of maize kernels at three different stages of swelling or germination (0, 3, and 96 hours) were incubated for 4.5 hours at +28° C in a phosphate buffer, pH 7.0, with or without the addition of 100 mg tryptophane per litre. After incubation, the kernels

Table 2. Amount of free auxin extractable during 1 hour at $+2^{\circ}\text{C}$ from maize kernels at different stages of swelling. Extractions were made after incubation of the kernels for 4.5 hours at $+28^{\circ}\text{C}$, in a phosphate buffer, pH 7.0, with or without the addition of 100 mg tryptophane per litre.

No. of hours during which the kernels had swelled before treatment	Treatment. With tryptophane=+, Without tryptophane=—	WAE/1000 per kernel		Difference, expressed as WAE/1000 per kernel, between kernels with and without the addition of tryptophane
		Det.	Average	
0	+	11.21 12.87 13.13	12.4	
0	—	9.46 11.07	10.3	2.1
3	+	11.96 13.20	12.6	
3	—	8.86 8.86 9.72	9.1	3.5
96	+	2.14 2.43	2.3	
96	—	1.07 1.50	1.3	1.0

were well rinsed and carefully dried with filter paper. They were then ground in a mortar and extracted for 1 hour at $+2^{\circ}\text{C}$ with ether. The ether was changed once during this time. The results (see Table 2) show that extracts of kernels incubated with tryptophane contained more free auxin than extracts of kernels incubated without tryptophane. Thus the tryptophane constitutes an auxin precursor from which the maize kernels can form free auxin fairly rapidly. The tryptophane preparation used proved to be completely inactive in the *Avena* test and consequently contained no traces of indoleacetic acid.

Summary

1. The content of free and bound acid auxin was determined in maize kernels at different stages of swelling. In the present study free auxin means auxin extractable in 3 hours at $+2^{\circ}\text{C}$ with a change of ether at the end of each hour. Bound auxin means auxin extractable from the same material during the subsequent 45 hours at $+28^{\circ}\text{C}$. Within this period the ether is changed twice.

2. Dry maize kernels from the 1953 harvest, which had been stored in the laboratory from May 19 to Sept. 1, 1954, contained considerably more bound

auxin at the beginning of the storage period than they did at the end of it, although the content of free auxin remained unchanged. It has been demonstrated that this cannot be due to the fact that a reduction in the water content of the kernels during storage, resulted in a reduced yield in the extractions; this must be due instead to changes in the content of auxin precursors in the kernels.

3. The content of free auxin was consistently higher in extracts from maize caryopses that had swelled for 24 or 48 hours than it was in extracts of caryopses that had swelled for only 3 hours. In these it was consistently higher than in extracts of dry kernels. Concurrently with the increase in the amount of free auxin in the kernels during swelling, there was a decrease in the amount of bound auxin. In extracts of kernels that had swelled for 24 hours there was in all cases a smaller amount of bound auxin than in extracts of kernels swelled for 3 hours.

4. Results of auxin determinations in extracts of isolated embryos and endosperms were in accord with the view that, in the dormant caryopse, auxin precursors occur most abundantly in the endosperm, whereas the conversion enzymes, active during extractions, are located mainly in the embryo. During the process of swelling part of the auxin precursors are probably transferred to the embryo.

5. If maize kernels are incubated for 3 hours in an aqueous solution of synthetic indoleacetic acid, 100 mg per litre, the content of free auxin in the kernels rises very considerably at first. Within 24 hours, however, most of this free auxin is transformed into bound auxin.

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References

- Avery, G. S. Jr., Berger, J., & Shalucha, B.: The total extraction of free auxin and auxin precursor from plant tissue. — *Amer. J. Bot.* 29: 596. 1942 a.
 — Auxin content of maize kernels during ontogeny, from plants of varying heterotic vigor. — *Amer. J. Bot.* 29: 765. 1942 b.
 Avery, G. S. Jr., Creighton, H. B., & Shalucha, B.: Extraction methods in relation to hormone content of maize endosperms. — *Amer. J. Bot.* 27: 289. 1940.
 Bentley, J. A. & Housley, S.: Studies on plant growth hormones. I. Biological activities of 3-indolylacetaldehyde and 3-indolylacetonitrile. — *Journ. Exp. Bot.* 3: 393. 1952.
 Berger, J. & Avery, G. S. Jr.: Isolation of an auxin precursor and an auxin (indoleacetic acid) from maize. — *Amer. J. Bot.* 31: 199. 1944.
 Boysen Jensen, P.: Über eine Mikromethode zur quantitativen Bestimmung der Wachstumsstoffe der A-Gruppe. — *Planta* 26: 584. 1937.
 — Quantitative Bestimmung der beschleunigenden Streckungswachstumsstoffe in der sauren Fraktion der Ätherextrakte aus höheren Pflanzen. — *Planta* 31: 653. 1941.

- Dettweiler, C.: Über den Einfluss des Heteroauxins auf die Wuchsstoffbildung in höheren Pflanzen. — *Planta* 33: 258. 1942.
- Funke, H.: Über Hemm- und Wuchsstoffe des keimenden Maiskorns. — *Jahrb. wiss. Bot.* 91: 54. 1943.
- Gordon, G. A.: Occurrence, formation, and inactivation of auxins. — *Ann. Rev. Plant Phys.* 5: 341. 1954.
- Guttenberg, H. von & Lehle-Joerges, E.: Über das Vorkommen von Auxin und Heteroauxin in ruhenden und keimenden Samen. — *Planta* 35: 281. 1947.
- Haagen-Smit, A. J., Leech, W. D., & Bergen, W. R.: Estimation, isolation and identification of auxins in plant material. — *Science* 93: 624. 1941.
- Hatcher, E. S. J.: Auxin production during development of the grain in cereals. — *Nature* 151: 278. 1943.
- Hatcher, E. S. J. & Gregory, F. G.: Auxin production during the development of the grain of cereals. — *Nature* 148: 626. 1941.
- Hemberg, T.: Studies of auxins and growth-inhibiting substances in the potato tuber and their significance with regard to its rest-period. — *Acta Horti Berg.* 14: 133. 1947.
- Rooting experiments with hypocotyles of *Phaseolus vulgaris* L. — *Physiol. Plant.* 4: 358. 1951.
- Studies on the occurrence of free and bound auxins and of growth-inhibiting substances in the potato tuber. — *Physiol. Plant.* 7: 312. 1954 a.
- The relation between the occurrence of auxin and the rooting of hypocotyls in *Phaseolus vulgaris* L. — *Physiol. Plant.* 7: 323. 1954 b.
- The auxin balance during germination in untreated maize kernels and in maize kernels treated with indole acetic acid. — *Sv. Bot. Tidskr.* 48: 805. 1954 c.
- Hinsvark, O. N., Houff, Wm. H., Wittver, S. H., & Sell, H. M.: The extraction and colorimetric estimation of indole-3-acetic acid and its esters in developing corn kernels. — *Plant. Phys.* 29: 107. 1954.
- Juel, I.: Der Auxingehalt in Samen verschiedenen Alters sowie einige Untersuchungen betreffend die Haltbarkeit der Auxine. — *Planta* 32: 227. 1941.
- Kruyt, W.: A study in connection with the problem of hormonization of seeds. — *Meded. Bot. Lab. Univ. Utrecht.* Nr. 1. 1954.
- Linser, H.: Zur Methodik der Wuchsstoffbestimmung. II. Die Extraktion von Pflanzenmaterial. — *Planta* 29: 392. 1939.
- Overbeek, J. van, Vazquez, E. S. de, & Gordon, S. A.: Free and bound auxin in the vegetative pineapple plant. — *Amer. J. Bot.* 34: 266. 1947.
- Siegel, S. M. & Galston, A. W.: Experimental coupling of indoleacetic acid to pea root protein in vivo and in vitro. — *Proc. Nat. Acad. Sciences* 39: 1111. 1953.
- Sircar, S. M. & Das, T. M.: Growth hormones of rice grains germinated at different temperatures. — *Nature* 168: 382. 1951.
- Stehsel, M. L. & Wildman, S. G.: Interrelations between tryptophane, auxin, and nicotinic acid during development of the corn kernel. — *Amer. J. Bot.* 37: 682. 1950.
- Yamaki, T. & Nakamura, K.: Formation of indoleacetic acid in maize embryo. — *Sci. Papers Coll. Gen. Ed. Univ. Tokyo* 2, No. 1. 1952.

Passive Components in the Ion Absorption of the Plant.

I. The Zonal Ion and Water Absorption in Brouwer's Experiments

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The ion absorption of the plant from the medium has long been regarded as an active process associated with the metabolism in the root. During the most recent years, however, strong evidence has been presented from different quarters that the ion absorption of the plant is of a complex nature. Thus it has been established that ion diffusion from the medium to the root with subsequent adsorption to the cell walls and plasma together with mass flow of ions with the transpiration stream accounts for a considerable part of the ion uptake of the plant. Some of the ions which by diffusion or mass flow from the medium penetrate into the root are actively accumulated in the root tissues, above all in the vacuoles, while the other ions are carried passively further to the shoot with the transpiration stream or are pressed actively to the shoot by the bleeding mechanism. A valuable resumé of the more recent studies on the mechanism and different components of ion uptake has been made by Burström (1954).

Diffusion with adsorption. It has frequently been demonstrated that when excised roots are transferred to a more concentrated medium a rapid uptake of ions takes place immediately after the transfer. This phenomenon is currently interpreted as a diffusion of ions to the parts of the root which are freely accessible to ion diffusion from the medium. This first component of ion uptake includes also the subsequent adsorption of ions to electrically charged points in the cell walls and above all in the cytoplasm within the root. Here positive ions exhibit a higher adsorption than negative ones. If the root is placed in a weaker medium, on the other hand, ions from the root dif-

fuse back into the medium. In this discussion Hope and Stevens (1952) have coined the expression, »the apparent free space» (A.F.S.), as a designation of the proportion of a given root tissue which appears to reach the external concentration when placed in a solution. For further data concerning this problem the reader is referred to Hope and Stevens (1952), Butler (1953), Hope (1953), and Hylmö (1953).

Mass flow. The comprehension of the cell walls of the root and possibly also the cytoplasm to a certain extent being accessible to diffusion from the medium agrees well with the experimental data which establish that ions in mass flow are drawn by the transpiration stream from the medium through the root to the shoot. The author has been able to demonstrate increased uptake of calcium as well as chloride ions with increased water flow in three-week-old pea plants (Hylmö 1950, 1953). The rate of water transport was varied in four different ways, namely through 1. altered relative humidity, 2. darkness-light, 3. different root temperatures and 4. use of truncated plants. The ion uptake was shown to be dependent on the rate of water flow but independent of the manner in which the transpiration was varied. Reviews of older works on the significance of transpiration for the salt uptake of plants are given by Hylmö (1953) and Brouwer (1954). Some of the recent publications will be mentioned.

Petritschek (1953) was able to demonstrate by determination of the electrolyte content in the stems of Clematis bushes in nature that with the higher intensity of transpiration during the day considerably more ions were transported through the stems than during the night. In his survey of translocation of solutes in plants Huber (1953) has also accepted the transpiration stream as an ion transporter. Likewise Butler (1953) found a correlation between water flow and ion uptake in young wheat plants in water culture. Brouwer (1953 d, 1954) has also shown in his extensive investigations that the transpiration in peas and broad-beans affects the magnitude of the ion uptake. For peas Brouwer (1954, p. 306) found a rectilinear connection between water flow and anion absorption.

In comprehensive studies on maize, Salicornia and Suaeda, Oknina (1953) found that the transpiration stream accelerated the chloride uptake of the plant. The ion uptake was not completely dependent on the water flow for chloride uptake occurred simultaneously independently of the transpiration. Thus the water flow accounted for 20 to 60 per cent of the total chloride absorption from a dilute solution of potassium chloride during normal transpiration.

In a recently published work Mes (1954) found that the leaves' excretion of salts (P^{32}) in rain and with soaking increases strongly after periods of intensive transpiration. The excretion of the leaves comprised a considerable part of the mineral storage of the plant.

Additional evidence is found in works where the authors themselves have not drawn the conclusion of the connection between water flow and ion uptake. Vöchting (1953, figure 6 and table 14) found in young maize plants that the total zinc content per plant did not increase during the first six days after the sowing despite a rather vigorous root development. On analysis 12 days later, however, the zinc content in the plant had been doubled and after 21 days it had increased five-fold. The author's interpretation of this phenomenon is the following: the roots themselves absorb none or very little zinc and first during the 7—12 days after transpiring leaves have developed is zinc drawn into the plant with the water flow. With the very greatly diluted medium employed (15 μM -Zn) the diffusion in A. F. S. can hardly be expected to be measurable.

Hansson and Biddulph (1953) investigated the migrations of the rubidium (Rb^{86}) and phosphate (P^{32}) ions in young bean plants during different periods of the day and night and found that the ion transport to the shoot was appreciably higher during the day than during the night. They demonstrated later that the fluctuation in the ion transport was not due to a 24-hour-rhythm but that light during the night as well as light during the day had the same effect and they concluded that the increased ion transport to the shoot was connected with the light. A determination of the water flow in Hansson and Biddulph's experiment would probably have shown that the transpiration was the primary factor.

Accumulation. Building further on Strugger's (1942) assumption of ion transport in the cell walls of the leaves the author (Hylmö, 1953) believes that also the cells in the root are surrounded by an aqueous medium containing dissolved salts. The root cells are assumed to be immersed in a nutrient medium from which the cell individually, actively accumulates ions to the cytoplasm and vacuole analogically to the behaviour of the unicellular submerged alga. Thus the root actively absorbs ions only for its own requirement. For the shoots' salts the root is only a transport route through which ions are passively borne with the help of the transpiration stream. Nevertheless the phenomenon which we observe in excised roots as bleeding is also an active absorption, where the punctured, still living xylem cells accumulate ions (test-tube hypothesis, Hylmö, 1953). Mes (1954) also regards the cell wall as a transport route for salts in exudation through the epidermis.

Brouwer's experiment

Brouwer (1953 d, 1954) has verified the author's experimental results and found that the ion uptake in intact plants increases markedly with increasing transpiration. On the other hand, Brouwer proposes an interpretation of the

physiologic background to the observed relationship which deviates strongly from the author's explanation. The author has previously (Hylmö 1953, pp. 381—386) discussed in detail the question of whether the transpiration stream directly or only indirectly affects the ion uptake. Indirect action of the transpiration stream would imply that the root first actively accumulates all the ions which pass the root. The transpiration stream is thereby conceived as only accelerating the active absorption by further transport of previously accumulated ions. Available experimental material in the author's opinion strongly favours the theory that the ions are thus passively drawn through the root to the shoot in mass flow independently of the metabolism in the root. Contrary to this Brouwer (1954, p. 306) believes that the salt uptake at high as well as at low suction tension from the shoot is a fully active process and that there is no reason to suppose a passive transport of ions in the transpiration stream for the materials used.

This paper is completely devoted to an analysis of Brouwer's (1954) experimental data, which will be presented here diagrammatically according to the method the author has previously applied (Hylmö 1953). Brouwer's experiments agree very well with mine and in my opinion they provide new and valid evidence that the ion uptake of the root is of a complex nature and that the transpiration component is passive and independent of the metabolism in the root.

Brouwer denies the occurrence of passive ion uptake, in spite of the fact that he has not divided up the ion passage into the root into its components. Nevertheless Brouwer's material permits such a division. His experiments are of so high class that they are worthy of a detailed revision. His technique is interesting and should in the future be able to give further insight into the role of the root in the water and mineral supply of the plant.

Method. Brouwer (1954) used five- to seven-week-old *Vicia faba* plants grown in tap water and thus deficient in salts. Water flow and ion absorption were determined for individual root branches divided into 3-cm zones according to a method which is based on Brewig's (1937) micropotometer technique. The root-tip zone is designated zone I and the successive 3-cm zones II, III, IV and V.

Passive components in the ion uptake

When Brouwer employed 2,4-dinitrophenol (DNP) as the inhibitor the rate of water flow was not affected but, on the other hand, the ion uptake of the root was. The experiment with chlorides from Brouwer's table IX (1954) has been plotted in figure 1 A. Water and ion passage could not be determined

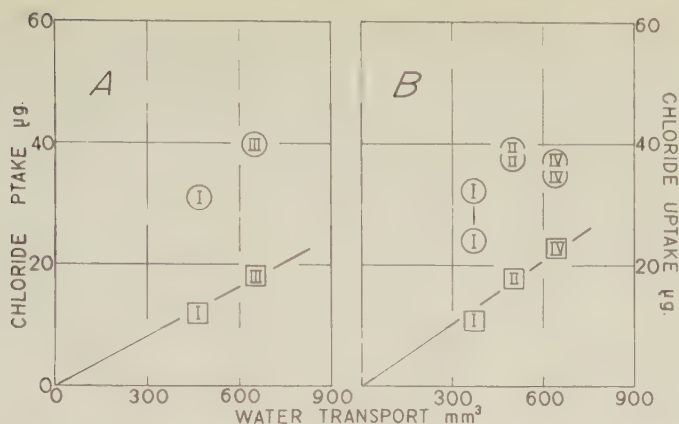


Figure 1. The influence of 10^{-5} M-2,4-dinitrophenol (A) and of oxygen deficiency (B) on the water and chloride uptake of the different root zones at high suction tension in the vessels. Uptake per 3 cm of root length during 24 hours from a medium of 5 mM-calcium chloride. Roman numerals designate the root zones counted from the root tip.

A. Circles denote without DNP, squares with DNP. From Brouwer (1954, table IX A).

B. Circles denote aerated roots, squares without air. From Brouwer (1954, table X).

simultaneously in the same zone but Brouwer has recorded the water passage in every other zone and the chloride uptake in the intervening ones. By interpolation I have calculated the water influx into the zone where only the ion uptake has been determined. In view of Brouwer's very regular experimental values and the good agreement between different experiments this procedure gives a good idea of the rate of the water flow. DNP inhibited a considerable part of the ion passage from the medium to the root. Similarly Brouwer (1954) found in another experimental series a strongly reduced ion uptake in oxygen deficiency (figure 1 B).

It is remarkable that both when the metabolism is disturbed with DNP and through decreased oxygen tension the residual ion uptake of the inhibited root zones is directly proportional to the water flow. The regression lines for the relationship water/ion passage point in both cases almost towards the origin, which indicates that without water flow no ion uptake occurs. With the DNP concentration employed the salt accumulation is arrested (Robertson, Wilkins and Weeks 1951, Lundegardh 1952 and Butler 1953). Oxygen deficiency has also been shown to inhibit the active ion uptake. The residual uptake demonstrated here represents the passive components in the ion uptake, namely, diffusion and mass flow. The diffusion component for the negative chloride ions, which are little adsorbed to the structures of the root, can be expected to be weak as in pea roots (Hylmö 1953, p. 373) and wheat

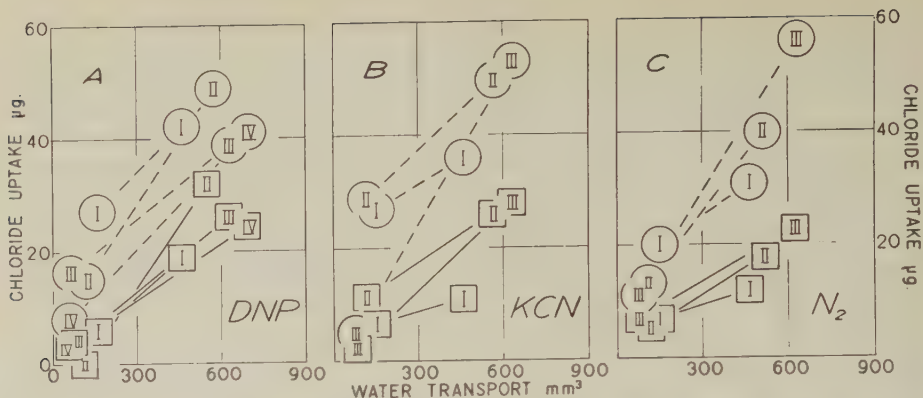


Figure 2. The influence 10^{-3} M-2,4-dinitrophenol (A), of $5 \cdot 10^{-6}$ M-potassium cyanide (B), and of nitrogen bubbling through the medium (C) on the chloride uptake of the different root zones at low and at high water uptake. Uptake per 3 cm of root length during 24 hours from a medium of 5 mM-calcium chloride. Roman numerals designate the root zones counted from the root tip. Circles denote without inhibitors, squares with inhibitors.

From Brouwer (1954, table VI).

roots (Butler 1953) and to be negligible in comparison to the mass flow. In all the series treated here Brouwer started the readings four hours after the plants were transferred to the experimental medium. An equilibrium is thereby attained with the medium and diffusion with subsequent adsorption is not included in the reported values on ion uptake. Brouwer's technique of rinsing with distilled water after each step in the experiment may also be assumed to diminish the root's content of free ions in the cell walls.

In another series of experiments Brouwer (1954, table VI) has compared the chloride uptake to the root during the use of different inhibitors with weak and strong water flow. In these experiments the rate of water flow has not been determined, but the author has calculated from those of Brouwer's experiments where »low» and »high» water uptake is reported probable values for the individual zones and employed these in the graphic representation (figure 2). Brouwer's different series agree rather well in regard to the water flow and even if there should exist appreciable deviations from the normal in individual points in the experiments accounted for here, they would have very little effect on the general impression. The essential factor is that the water flow is higher during »low» than »high» water uptake. Only the numerical value of the influx coefficient should be noticeably influenced.

These experiments with different inhibitors, DNP and KCN, and with nitrogen bubbling yield concordant results (figure 2). The ion uptake is partially arrested. The residual, uninhibited ion uptake is directly proportional to the

water flow and extrapolation of the regression lines almost meets the origin. Without water flow there is no ion uptake when an inhibitor has been added. The nitrogen bubbling has not repressed the ion uptake fully so effectively, but a certain accumulation to the cells of the root persists even during low water flow. These series (figure 2) complement extremely well the foregoing in that they show the variation within one and the same root zone, while the preceding ones (figure 1) treat the ion passage to different zones. The passive ion transport with mass flow is thus independent of the varied metabolic activity of the zones and only conditioned by the rate of water flow.

The fact that the ion uptake component which is unaffected by DNP, KCN, and oxygen deficiency is directly proportional to the rate of water flow constitutes conclusive evidence that chloride ions are passively drawn with the transpiration stream from the medium through the root to the shoot, independently of the metabolism of the root. It does not support the theory that the ions are transported through an endodermal barrier and actively secreted to the xylem, unless we are willing to assume that the ion transport here is mediated by a metabolism which is not repressed by the inhibitors employed or by anaerobic medium.

Remarkable evidence that the passive ion components are independent of the varied activity of the zones is also found in other series (Brouwer 1954, figure 6). Here is encountered the interesting circumstance that the regression lines for the controls without sucrose fall in zonal order (figure 3). Zone I has with the same rate of water flow greater chloride absorption than zone II and the latter zone greater than the older zones III and IV (III falls somewhat below IV). Interpreted according to Hylmö (1953) this shows that the accumulation (including diffusion, which is, however, assumed to be low and to constitute a very small part of the independent ion uptake) is greatest in the root tip and the zone adjacent thereto. It is most remarkable that the regression lines have largely the same slope, which implies that the water passage has the same effect on the ion uptake in all zones. (The regression line for zone I can only be drawn with difficulty as the dispersion in the water flow is insignificant.) The concentration of the true transpiration stream is practically the same in all the zones. It is 1.2 mM-Cl⁻ in zone II, 0.8 in zone III and 1.2 in zone IV when the concentration of the medium was 10 mM-Cl⁻.

Practically the same picture is obtained in another experimental series (figure 4), where the regression lines of the root zones fall in the same order. For the zones I, II and IV the lines have the same slope. Zone III has one strongly divergent point, where the ion uptake with weak water flow is very low. The concentration of the true transpiration stream is for the zones I II and IV 0.5, 0.8 and 0.8 mM-Cl⁻, respectively. For zone III the corresponding value is 2.0 mM. The medium had a concentration of 10 mM-Cl⁻.

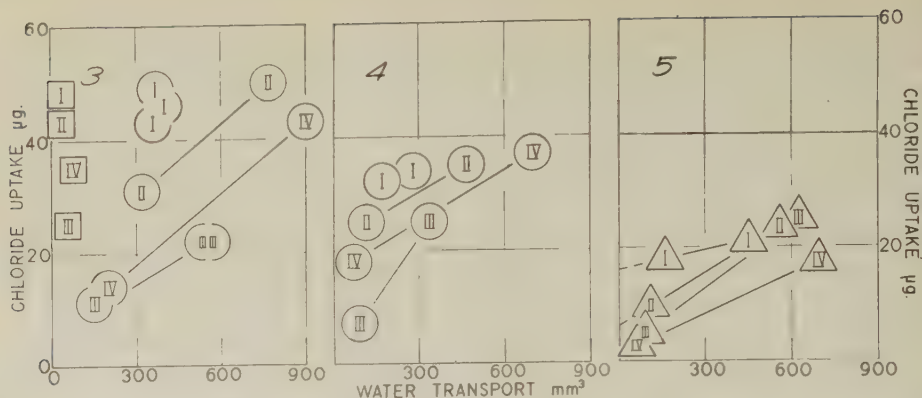


Figure 3. The water and chloride uptake of the different root zones, at low and high suction tension with and without an osmotic counter suction of sucrose in the medium. Uptake per 3 cm of root length during 24 hours from a medium of 5 mM-calcium chloride. Experimental period 16 hours. Squares denote with sucrose (2.5 atmospheres), circles without. From Brouwer (1954, figure 6).

Figure 4. The water and chloride uptake of the different root zones at low and high suction tension. Uptake per 3 cm of root length during 24 hours from a medium of 5 mM-calcium chloride. From Brouwer (1954, table VIII).

Figure 5. The relationship between the water flow and the chloride uptake arrested by inhibitors or oxygen deficiency at low and high suction tension. Uptake per 3 cm of root length during 24 hours from a medium of 5 mM-calcium chloride. Each point is the mean value of 4 determinations (for zone IV only 2 determinations). From Brouwer (1954, tables VI and VII). Inhibitors employed were 10^{-5} M-DNP, $5 \cdot 10^{-6}$ M-KCN, N_2 bubbling and non-aeration of the roots.

It should be observed that in the experiments on which the figures 3 and 4 are based Brouwer has determined the water flow in the one zone and the ion uptake in the other as for figure 1. Therefore the rate of water flow here can with fairly great certainty be interpolated forward for the chloride zones. When in the figure 2 the regression for the zones exhibits a greater variation this can be explained by the fact that in these series the rate of water flow was not determined at the same time as the chloride uptake. As has already been mentioned the dotted water values may thereby be expected deviate somewhat from the actual values.

Effect of sucrose

Brouwer (1954, figure 6) considers his strongest evidence that the ion uptake which is dependent on the water flow is metabolically affected to be the experiments he carried out with an increased osmotic value of the medium.

In spite of the strongly diminished water flow as a result of the addition of sucrose the ion uptake remained at a high level. According to Brouwer this is an indication that water and ions reach the root independently of each other. When sucrose in spite of a very weak water flow produces a high ion uptake (figure 3) it must, however, be kept in mind that sucrose has not only an osmotic effect but also it has long been known to increase ion accumulation in the excised roots just as in tissue parts. Brouwer was unsuccessful with mannitol as the osmotic agent, which is known to have no effect on the metabolism, and was forced to turn to sucrose. In the author's opinion this move was disastrous, because sucrose through its energy addition may be assumed to have enhanced metabolism in the roots and thereby the root cells' own accumulation of ion. Brouwer states that the mannitol solution only slightly reduced the water passage. In hitherto unpublished experiments the author has demonstrated in pea plants that mannitol strongly reduces the transpiration and that the ion uptake remains the same as with a corresponding water flow when the transpiration is decreased by keeping the plants in moist air or in darkness.

The best evidence that the effect of sucrose in this case is not only osmotic but also metabolic is furnished by Brouwer himself (1954, figure 7). Here the ion uptake in zone III is increased two-fold on the addition of sucrose in comparison to that with low water passage without sucrose. Repetition of this experiment gave the same results. A reduction of the water flow through elevation of the counter pressure of the medium according to Brouwer's reasoning should leave the salt uptake undisturbed and under no circumstances as here increase the ion uptake. The increased ion uptake is quite certainly produced by the energy addition which in the form of sucrose is supplied to the general metabolism of the root.

Active components in the ion uptake of the plant

In Brouwer's series with broad-beans the magnitude of the active components in the ion uptake of the plant is also enlarged with increased water flow. An example of this is given in figure 5, where the author has calculated the mean values from a series of experiments which Brouwer performed with different inhibitory agents. The active components have been regarded as constituting the difference between the ion passage into the root without and with an inhibitor. The ion uptake sensitive to inhibitory agents is greatest in the root tip and decreases basally. The water flow affects the ion uptake in zone I less than in the other zones. This indicates that there is a greater accumulation of ions in the growing cells of the root tip. In the basal zones the

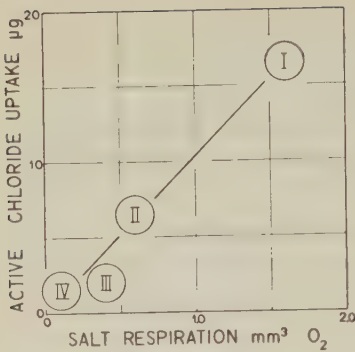


Figure 6. *The relationship between the salt respiration and the active chloride uptake of the root zones.* The salt respiration is calculated as the difference between the respiration in 10 mM-KNO₃ and in distilled water and expressed as the consumed volume of oxygen in mm³ per hour and zone. From Brouwer (1954, figure 10). The active chloride uptake is represented by the part of the ion uptake repressible by inhibitors in plants without water passage. The uptake is expressed in μg chloride per root zone during 24 hours (cf. figure 5).

active ion uptake probably partly represents the bleeding stream towards the shoot. Without a simultaneous analysis of the storage of ions in the root and in the shoot the site of accumulation cannot be determined. Analysis of loss of ions from the medium as is the case here cannot, of course, give a picture of the fate of the ions in the plant and does not permit a true evaluation of whether the demonstrated active uptake in the basal zones is a bleeding stream or an accumulation in the root cells due to the accessibility of the ions through mass flow to the more internally located cell layers of the root.

Brouwer (1954, p. 283) believed that he had found evidence that the enhanced ion uptake with increased water flow is an active process, when he inhibited in plants with high water flow a salt uptake which was greater than the total absorption in plants with low water flow. In peas the root's own accumulation of chloride ions rises markedly with increased water passage (Hylmö 1953, figure 6). We may assume that the same condition exists in the broad-bean root and that the demonstrated elevation of active ion uptake with increased water passage (figure 5) is composed partly of accumulation in the root. The root has thus two active components of ion uptake, one which is independent and one which is dependent on the water passage. For other kinds of ions such as phosphate in broad-beans (Brouwer 1954) and calcium in peas (Hylmö 1953) it has been shown that the root's own uptake of ions is fairly independent of the rate of water flow. It should be observed that the roots were supplied with phosphate and calcium in the nursery prior to the experiment but not with chloride.

It is noteworthy that the salt respiration proved by Brouwer (1954, figure 10) is independent of the rate of water flow, while the chloride uptake sensitive to inhibitors (figure 5) is strongly dependent on the water flow. The salt respiration is considered by Brouwer to constitute the difference between the total respiration in 0.01 M-KNO₃ solution and in distilled water. The salt respiration corresponds in zonal order I to IV to an oxygen consumption of

1.6, 0.6, 0.4 and 0.1 mm³ per hour and zone. Strangely enough the relations are the same between the active ion uptake of the zones, calculated as the regression lines' intersections of the y-axis, when the water passage is thus non-existent. This ion uptake (figure 5) is in zonal order 16.5, 6.5, 2.0 and 1.5 µg Cl⁻ per 24 hours. It is conceivable that the salt respiration demonstrated in Brouwer's experiment concerns only the root's own accumulation of ions in the outer cell layers and not the more central tissues or the bleeding accumulation. The relationship between the salt respiration and the active ion uptake at zero water passage implies that 15 mols ¹/₄ O₂ are required for the transport of 1 mol Cl⁻ (figure 6). Future investigations must decide whether this demonstrated parallelism between salt respiration and ion accumulation is real or only caused by chance.

Influx coefficient

The concentration of the true transpiration stream from the medium to the root in relation to the concentration of the medium, the influx coefficient *i*, in the experiments described here is much lower than in the author's observations in peas, which independent of the concentration of the medium in regard to calcium as well as chloride ions had a value of c. 0.55 (Hylmö 1953). In the broad-bean the influx coefficients for chloride in the recalculated experiments without any inhibitor have the following values:

Figure no.	Zone I	Zone II	Zone III	Zone IV
2 A	0.14	0.21	0.16	0.14
2 B	0.09	0.14	0.24	—
2 C	0.10	0.18	0.23	—
3	—	0.12	0.08	0.12
4	0.05	0.08	0.20	0.09

In view of the fact that each value in the foregoing table is calculated from only two points the dispersion is surprisingly small. For a true determination of the magnitude of the influx coefficient, however, a large material is required. There does not appear to be hardly any certain variation between the different zones. The medium thus runs in in a diluted state also in the broad-bean. The dilution is so great that the true transpiration stream has only 5 to 24 per cent of the chloride concentration of the medium. From an earlier investigation (Brouwer 1953 d) the influx coefficient in *Vicia faba* can be calculated to a somewhat higher value or 0.38 and 0.50 for chlorides. These values, which are valid for a larger part of the root than the zones in the foregoing table, are in better numerical agreement with the author's influx coefficients for peas.

Pressure drop through friction and filter effect

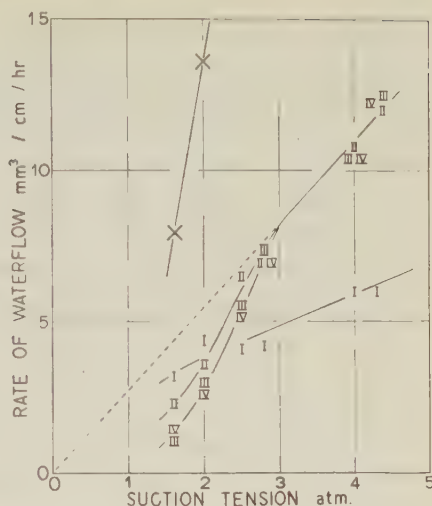
Brouwer has unfortunately based the interpretation of his results on Brewig's (1937) assumption that the resistance of the root to water flow is altered with the suction tension from the shoot. Both Brewig (1937) and Brouwer (1953 a, b, c; 1954) found that with low water passage the water entered mainly through the apical zones; when the water flow was increased the basal zones took care of the increase in water flow so that these basal zones during strong transpiration are responsible for the principal water maintenance of the plant (figure 7). Rosene (1941) had earlier verified Brewig's experiment but she opposed the use of the idea of resistance. Rosene's »objection to such a conclusion is that no data are available in the literature which identify the components of the »resistance» as in the case in the ohmic resistance of an electric current». Brewig's conclusion that the resistance of the root is altered with the intensity of the transpiration has previously been criticized also by Kramer (1949, p. 114) and Hylmö (1953, p. 398). Brouwer (1954, p. 308), nevertheless, builds further on Brewig's theory and concludes that even »the anion uptake at high suction tension in the xylem vessels is as much facilitated as the water uptake, or in other words the conductivity for water and anions is increased to the same degree».

The occurrence of a greater resistance to flow with a greater velocity of the current of water in the plant has been discussed by Dixon (1914). In the author's opinion it is not in accordance with the hydrodynamic facts to speak about any change in resistance, conductivity or permeability to water movement.

Brouwer (1954, figure 2) does not report the direct relationship between water flow and suction tension but instead the relationship between the constructed conductivity value and suction tension. In an earlier paper Brouwer (1953 a, figure 6) gives the equally constructed resistance value instead of the water flow. A recalculation of these data of Brouwer's reveals an interesting picture of the relationship between water passage and suction tension in the xylem (figure 7). The total water passage in the zones I to and including IV, i.e., the lower 12 cm of the root, is directly proportional to the suction tension over a large area, indicating that the water passage ceases at c. 1 atmosphere. Without direct measurements also at lower suction tension it is, however, impossible to state whether the relationship is rectilinear also at low pressure differences. At the higher suction tension of 4.0 and 4.3 atmospheres a lesser drop in the water passage is discerned.

At the lowest tested suction tension, 1.6 atmospheres, the zones I and II take care of the principal water supply. The water passage in zone I is only

Figure 7. The dependence of the water flow on the suction tension from the shoot in the root zones of *Vicia faba*. The values are determined at a steady state, the water uptake and the suction tension being constant for several hours. The suction tension in the xylem vessels was 1.6, 2.0, 2.5, 2.8, 4.0 and 4.3 atmospheres. The individual zones are designated with Roman numerals, the total uptake of the examined zones with crosses. From Brouwer (1954, figure 2).



slightly increased with elevated suction tension but quite increased in the other zones. At the higher pressures the water flow is of the same rate in the zones II, III and IV. The flow-pressure curves for these last mentioned zones show a remarkable agreement with the diagrams Erbe (1933) and Pisa (1933) produced in the determination of the pore-size distribution of ultrafilters, when water is forced through a membrane displacing air or isobutylic alcohol. An S-formed curve passes over into a straight line. Erbe (1933) gives the following interpretation of the flow-pressure curve. At a certain constant pressure on the filter the largest pores are opened for water. When the pressure is increased another group of smaller pores is opened, which is evidenced by a corresponding rather than a proportional increase in the flow through the filter. The discontinuous increase in the flow proceeds until all the pores are opened for water. With further increased pressure the increase in the water flow becomes proportional to the increase in pressure in accordance with Hagen-Poiseuille's law.

In Brouwer's experiment water does not replace air or another solution but water. The good agreement with Erbe (1933) and Pisa (1933) indicates that probably in the root water flows first through the coarser microcapillaries and that only after increased pressure is the water in the finer microcapillaries also set in motion. It should thus be possible to employ Brouwer's micropotometer technique for the determination of pore size in the paths the stream passes from the medium to the xylem. In this manner (Cantor's law) the pore diameter in the broad-bean root can be calculated to c. 15–40 μ . This should imply that with the employed suction pressure in the cellulose cell

walls only the coarser interfibrillar capillaries are utilized for water transport but not the finer interstices postulated by Frey-Wyssling (1948) between the micellar strands within the microfibrils.

According to Hagen-Poiseuille's law the rectilinear part of the curve should point toward the origin, which is also the case (figure 7). When the water flow is set in motion first after a certain suction pressure is attained, this may be explained by the fact that adsorption forces in the capillaries must be overcome before the water flow is started.

The relatively more rapid flow into the apical zones with low suction tension is probably due to the greater frequency of coarser pores in their cell walls. Apparently the microcapillaries in the cellulose walls are gradually closed with increasing age. The same result may, however, also be expected if the distance the water flow passes through the pores is shorter in the apical zones. Even bleeding may conceivably enhance the water passage in the apical zones.

Zone I diverges markedly from the others in regard to the position of the regression line (figure 7). The explanation lies certainly in differences in the anatomic structure. In the apical zone of the broad-bean root the tissue is not yet completely differentiated and open vessels are only developed to a limited extent. The water flow has to pass cell walls, cytoplasm and vacuoles not only radially but also longitudinally, whereby pressure losses through friction arise with more rapid flow. In the basal zones the water only has to pass the cell layers radially on the way to the xylem strands. The suction tension in zone I is thus not the same as in the other zones. The suction tension in zone I can be calculated to c. 1.8 atmospheres, when the suction tension of the shoot is 2.5 atmospheres and to c. 2.4 atmospheres when the suction tension is 4.3 atmospheres.

Brewig (1937) and Brouwer (1954) place according to Brieger (1929):

$$\text{water uptake} = k (S_{\text{xyl}} - S_{\text{med}});$$

where S_{xyl} and S_{med} denote the suction tensions (D.P.D.) in the xylem and the medium respectively. This is in full accord with Hagen-Poiseuille's equation for the flow of liquids through a tube, where l is the length of the tube, r its radius, p the difference of pressure at the ends, η the coefficient of viscosity, the volume escaping per time,

$$V = \frac{\pi p r^4}{8 l \eta}.$$

Since within a root zone in Brouwer's experiments the expression $\frac{\pi r^4}{8 l \eta}$ is constant the rate of flow becomes directly proportional of the difference of pressure:

$$V = k p.$$

Brouwer in conformity with Brewig calls k in these equations the conductivity of the root. When they attained values as in figures 7 over the water influx of the zones with high and low suction tension, they were forced to assume that the conductivity of the root is altered through impulses from the shoot disregarding Erbe's (1933) contention that Hagen-Poiseuille's law is valid first after all pores are utilized for water transport, after a certain suction tension has been reached. Brewig (1936) and Brouwer (1954) further assume that the suction tension is the same in the different zones, and they do not take into consideration the rising pressure drop through friction with increased rate of flow in the microstructures in the cell walls and plasma of the apical zone where xylem strands are not fully developed.

Summary

A revision is made of Brouwer's data on the zonal water and ion uptake of the broad-bean root. The plant's chloride uptake is shown to be of a complex nature. When through the addition to the medium of 2,4-dinitrophenol or potassium cyanide or through nitrogen bubbling the metabolism of the root is disturbed, a part of the ion uptake is inhibited. The residual ion uptake is directly proportional to the water passage, indicating that the ions are passively transported through the root in mass flow with the transpiration stream. The passive ion component is directly proportional to the water passage both when the same root zone is observed with different suction pressures and when different zones are compared with each other under the same suction pressure. On the other hand, the apical zones exhibit a higher active component of ion uptake repressible by inhibitors than the basal zones. Even the active ion component is partly dependent on the water flow.

In experiments with the addition of sucrose to the medium the water flow could be greatly limited through osmotic counter pressure without a corresponding inhibition of the ion uptake. In many series, however, the sucrose addition appreciably enhanced the total ion uptake of the strongly deficient plants. For this reason the sucrose through a supply of energy must be assumed to increase the metabolism of the root and the accumulation of ions. The addition of sucrose is not shown to affect the relation between the water flow and the passive components in the ion uptake.

The value of the influx coefficient (the relation between the concentration of the true transpiration stream and the concentration of the medium) was c. 0.1—0.2 for chlorides.

Brouwer's conception of impulses from the shoot regulating the conductivity of the root for water and ions is rejected. The relation between suction

tension and water flow is shown in the root zones instead to agree with laws well known from among other things animal tissues for flow through filters and ultrafilters. The pores, through which the water flow passes in the broad-bean root, are calculated to have a diameter of c. 15—40 μ .

Brewig's equation referred to by Brouwer is shown to be a derivation of Hagen-Poiseuille's law. This law is demonstrated to be valid in the root as in other ultrafilters first at higher pressures after the fluid in all pores has been set in motion. Due to the fact that the xylem vessels in the root tip are not fully developed the suction tension of the shoot during intensive transpiration is strongly reduced by pressure drop through friction.

References

- Brewig, A.: Die Regulationserscheinungen bei der Wasseraufnahme und die Wasserleitungsgeschwindigkeit in *Vicia Faba*-Wurzeln. — *Jahrb. wiss. Bot.* 82: 803. 1936.
- Brieger, F.: Untersuchungen über die Wasseraufnahme ganzer Pflanzen. — *Jahrb. wiss. Bot.* 69: 295. 1928.
- Brouwer, R.: Water absorption by the roots of *Vicia Faba* plants at various transpiration strengths. I. Analysis of the uptake and the factors determining it. — *Proc. Kon. Nederl. Ak. Wet. C* 56: 106. 1953 a.
- II. Causal relation between suction tension, resistance and uptake. — *Proc. Kon. Nederl. Ak. Wet. C* 56: 129. 1953 b.
- III. Changes in water conductivity artificially obtained. — *Proc. Kon. Nederl. Ak. Wet. C* 57: 68. 1953 c.
- Transpiration and anion uptake. — *Proc. Kon. Nederl. Ak. Wet. C* 56: 639. 1953 d.
- The regulating influence of transpiration and suction tension on the water and salt uptake by the roots of intact *Vicia Faba* plants. — *Acta Bot. Neerl.* 3: 264. 1954.
- Burström, H.: Mineralstoffwechsel. — *Fortschritte Bot.* 16: 269. 1954.
- Butler, G. W.: Ion uptake by young wheat plants. II. The "Apparent Free Space" of wheat roots. — *Physiol. Plant.* 6: 617. 1953.
- Dixon, H.: Transpiration and the ascent of sap in plants. — London 1914.
- Erbe, F.: Die Bestimmung der Porenverteilung nach ihrer Grösse in Filtern und Ultrafiltern. — *Kolloid-Zeitschrift* 63: 277. 1933.
- Frey-Wyssling, A.: Submicroscopic morphology of protoplasm and its derivatives. — Amsterdam 1948.
- Hanson, J. B. & Biddulph, O.: The diurnal variation in the translocation of minerals across bean roots. — *Plant Physiol.* 28: 356. 1953.
- Hope, A. B.: Salt uptake by root tissue cytoplasm: The relation between uptake and external concentration. — *Austral. J. biol. Sci.* 6: 396. 1953.
- Hope, A. B. & Stevens, P. G.: Electric potential differences in bean roots and their relation to salt uptake. — *Austral. J. Sci. Res. B* 5: 335. 1952.
- Huber, B.: Vergleichende Betrachtung der pflanzlichen Saftströme. — *Naturwissensch.* 40: 180. 1953.
- Hylmö, B.: Transpiration och ionupptagning. — *Nordisk För. Fysiol. Bot. Medd.* 2: 7. 1950.
- Transpiration and ion absorption. — *Physiol. Plant.* 6: 333. 1953.
- Kramer, P. J.: Plant and soil water relationships. — New York 1949.

- Lundegårdh, H.: Properties of the cytochrome system of wheat roots. — *Arkiv f. Kemi* 5: 126. 1952.
- Mes, M.: Excretion (recretion) of phosphorus and other mineral elements by leaves under the influence of rain. — *S. African J. Sci.* 50: 167. 1954.
- Oknina, E. Z.: Entrance of salts into a plant from nonequilibrated solutions. — *Acad. Nauk. S.S.S.R.* 8: 1: 354. 1953.
- Petritschek, K.: Über die Beziehungen zwischen Geschwindigkeit und Elektrolytgehalt des aufsteigenden Saftstromes. — *Flora* 140: 345. 1953.
- Pisa, M.: Versuche zur Porenstatistik und Siebwirkung bei Ultrafiltern und tierischen Membranen. — *Kolloid-Zeitschrift* 63: 139. 1933.
- Robertson, R. N., Wilkins, M. J. & Weeks, D. C.: Studies in the metabolism of plant cells. IX. The effects of 2,4-dinitrophenol on salt accumulation and salt respiration. — *Austral. J. Sci. Res., B*, 4: 248. 1951.
- Rosene, H. F.: Control of water transport in local root regions of attached and isolated roots by means of the osmotic pressure of the external solution. — *Amer. J. Bot.* 28: 402. 1941.
- Strugger, S.: Der aufsteigende Saftstrom in der Pflanze. — *Naturwissenschaft.* 31: 181. 1943.
- Vöchting, A.: Über die Zinkaufnahme von *Zea Mays* L. und *Aspergillus niger* v. Tiegh. in Einzelkultur und in Mischkultur. — *Ber. schweiz. bot. Ges.* 63, 103. 1953.

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Le métabolisme des acides organiques chez le *Bryophyllum* (Crassulacée)

I. Oxydations respiratoires et fixation de l'anhydride carbonique à l'obscurité par β -carboxylation, en fonction de la tension d'oxygène

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A. Introduction

L'étude du métabolisme des plantes grasses a été renouvelée par trois séries de recherches, l'une portant sur les échanges gazeux, l'autre sur l'activité enzymatique d'extraits végétaux, la troisième sur les acides organiques.

L'étude de l'acidité des sucs des plantes a permis de faire la jonction entre les manifestations externes du métabolisme et les propriétés enzymatiques attribuées aux tissus. Le cycle nyctéméral des variations de l'acidité des feuilles des Crassulacées ne pouvait s'expliquer par la seule succession des phénomènes prédominants selon le moment : oxydations pendant la nuit, photosynthèse pendant le jour.

Thomas (1949, 1951), a introduit dans l'interprétation de l'accumulation nocturne des acides organiques par les feuilles des Crassulacées, l'hypothèse de la fixation de l'anhydride carbonique par une réaction de β -carboxylation analogue à celle que Wood et Werkman ont mis en évidence chez les Bactéries, réaction dont la généralité a été reconnue dans les tissus animaux et végétaux (voir Wood, 1951; Ochoa, 1951, 1953; Truhaut, 1953).

En ne considérant que les échanges de gaz et les variations de l'acidité

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des feuilles de Crassulacées, tels que Thomas les a exposés, au cours de l'analyse des travaux effectués sur ce sujet depuis de Saussure et d'après les résultats des recherches qu'il a poursuivies avec ses élèves (Thomas et Beevers, 1949; Thomas et Ranson, 1954), on peut dégager les conclusions suivantes qui s'appliquent plus spécialement aux feuilles des Bryophyllum et des Kalanchoë. (Voir également Wolf, 1949).

A l'obscurité, les feuilles des Crassulacées respirent et, simultanément, fixent CO_2 , qu'il s'agisse du CO_2 libéré par les oxydations respiratoires ou du CO_2 de l'air. Il résulte de cette fixation par β -carboxylation, un enrichissement des tissus en acides organiques, notamment en acide malique.

La fixation de CO_2 et l'enrichissement en acides ne dure que peu de temps, une douzaine d'heures environ dans les conditions ordinaires. Après cette période intervient une libération de CO_2 dont l'origine est double : oxydations respiratoires habituelles des glucides et décarboxylation entraînant un appauvrissement en acides. La décarboxylation des acides peut être distinguée des oxydations respiratoires habituelles. Elle intervient alors que les tissus sont encore riches en glucides (Pucher et coll., 1947 c) et diffère de ce fait de l'oxydation finale des acides organiques caractéristique des feuilles carencées en glucides (Moyse, 1950). De plus, elle s'accompagne d'après Pucher et coll. (1947 c) et Vickery (1952 b) d'une resynthèse de l'amidon, en accord avec une hypothèse plus ancienne de Bennet-Clark (1933 a et b). Il s'agit alors d'un mécanisme de synthèse et non d'une simple dégradation.

Thomas et coll. (1949, 1954), pour mieux mettre en évidence la fixation de CO_2 externe par les feuilles de Bryophyllum à l'obscurité, ont placé ces feuilles en présence d'atmosphères enrichies en CO_2 . Ils ont mesuré l'absorption simultanée d' O_2 et de CO_2 , ainsi que l'accumulation d'acides, la β -carboxylation l'emportant alors sur la décarboxylation.

Si, dans l'air, le quotient respiratoire $\left(Q. R. = \frac{\text{vol. } \text{CO}_2 \text{ émis}}{\text{vol. } \text{O}_2 \text{ absorbé}} \right)$ est voisin ou égal à 0, par suite de la reprise du CO_2 , ce quotient devient négatif en atmosphère enrichie en CO_2 , l'absorption des deux gaz étant simultanée et celle de CO_2 s'ajoutant à la reprise directe du CO_2 qui est produit par les oxydations. Par contre, ce quotient est voisin de 1 quand la carboxylation a atteint sa limite et qu'elle s'équilibre alors avec la décarboxylation, ce qui se traduit par la stabilisation de la teneur des feuilles en acides. Thomas et coll. ont généralisé cette observation, étudiant différentes plantes grasses (Crassulacées, Opuntia, Kleinia) et vérifié l'influence de la température dont l'abaissement favorise la fixation de CO_2 et l'accumulation parallèle d'acides. Après Wolf, Bonner et coll. (1948), Thomas et coll. ont également étudié l'influence de la tension partielle de CO_2 sur la vitesse de l'accumulation d'acides. Si aux faibles tensions de CO_2 (0,1 à 1 pour cent), la fixation de CO_2

croît linéairement en fonction de sa concentration, la tension de 5 pour cent dans l'air paraît très favorable, les tensions élevées, moins favorables, peuvent provoquer des phénomènes fermentaires. Ces phénomènes fermentaires sont caractérisés par l'accumulation d'acides succinique, γ -amino-butyrique et parfois d'alcool. (Ranson, 1953).

Thomas et coll. ont également étudié les échanges de gaz et les variations d'acidité des feuilles de Crassulacées à la lumière, dans l'air enrichi ou non en CO_2 , questions sur lesquelles je reviendrai ultérieurement.

Ne considérant toujours que les variations des équilibres carboxylation-décarboxylation et leurs relations avec les oxydations cellulaires, à l'obscurité, il faut tenir compte des analyses biochimiques qui ont précisé quels étaient les acides dont les quantités étaient soumises à variations importantes chez le Bryophyllum.

Pucher et coll. (1947 b, 1948, 1949), Vickery (1952 a) ont confirmé que l'acide malique présente le plus fort accroissement de quantité, à l'obscurité, suivi d'une manière beaucoup plus faible par l'acide citrique, alors que les quantités d'acide isocitrique restent pratiquement inchangées. L'accumulation d'acide citrique diffère nettement de celle de l'acide malique. Elle continue lorsque se prolonge le maintien des feuilles à l'obscurité, alors que celle de l'acide malique décroît.

Thurlow et coll. (1948), Varner et coll. (1950), Stutz et coll. (1951) et Ranson (in Thomas et coll. 1954), utilisant l'anhydride carbonique radioactif $^{14}\text{CO}_2$, ont précisé son accumulation dans l'acide malique et, à un moindre degré, dans l'acide citrique, ainsi que dans d'autres substances (acides aspartique, glutamique, protéines, glucides etc. . .).

Si, du point de vue enzymatique, les modalités propres de la β -carboxylation effectuée par les feuilles de Bryophyllum ne sont pas encore bien précisées, il y a tout lieu de penser qu'elles sont très voisines de celles dont l'existence a été reconnue dans divers organes végétaux : feuilles, racines, tubercules, germinations, algues (Clendenning et coll., 1952).

Il s'agit soit de l'activité de l'enzyme malique (Conn et coll., 1949) soit d'une carboxylase oxalacétique dont l'action réductrice peut être complétée par celle d'un enzyme comme le triphosphopyridine-nucléotide réduit (TPN H_2) (Vennesland et coll., 1949, Vennesland, 1949), soit d'une carboxylase catalysant la fixation de CO_2 sur un phosphoryl-énolpyruvate (Bandurski et coll., 1953). Un enzyme tel que la deshydrogénase isocitrique, qui peut catalyser la fixation de CO_2 par l'acide α -cétooglutarique (Ceithalm et coll., 1949, Vennesland et coll., 1952) peut intervenir indirectement dans l'accumulation de l'acide citrique.

Les liens entre les oxydations cellulaires et la fixation de CO_2 par β -carboxylation peuvent être schématisés de la manière suivante, la β -carboxylation

intervenant en marge du déroulement du cycle de Krebs et portant principalement sur l'acide pyruvique ou un corps tricarboné voisin, avant l'entrée de ce corps dans le cycle tricarboxylique lui-même. Une autre voie latérale de β -carboxylation peut être supposée aboutir à l'enrichissement des feuilles en acide citrique.

L'accumulation d'acides est la résultante de 2 phénomènes continus : la carboxylation et la décarboxylation, et se manifeste lorsque le premier l'emporte en intensité.

Au contraire, l'amointrissement de l'acidité est du à la prédominance de la décarboxylation sur la réaction inverse.

En l'absence de CO_2 externe, le déroulement du cycle de Krebs, générateur de CO_2 , est nécessaire pour que l'acide pyruvique puisse être carboxylé. Il n'en est plus de même, théoriquement du moins, en présence de CO_2 externe.

Dans les deux cas, une partie de l'acide pyruvique est soustraite du cycle tricarboxylique.

L'accumulation d'acide citrique, par contre, paraît être obligatoirement tributaire du cycle, soit que le déroulement de celui-ci se trouve ralenti après la genèse de l'acide cis-aconitique, (ce qui suppose l'existence d'une réserve suffisante d'acide oxalacétique,¹ soit que l'acide citrique provienne indirectement de la carboxylation de l'acide α -cétooglutarique. Dans ce dernier cas, une partie de l'acide α -cétooglutarique est soustraite de la suite du cycle.

Plusieurs chercheurs, notamment Kraus (1883), Warburg (1886), Purjevitch (1893), Astruc (1903), Richards (1915), Wolf (1931—1932), ont déjà pensé que l'oxygène était indispensable à la genèse et surtout à l'accumulation des acides dans les tissus des plantes grasses. La composition même des acides, comparée à celle des glucides, et la valeur du rapport carbone-oxygène dans les molécules des deux groupes de substances, laissent supposer l'existence d'une relation directe entre les oxydations et l'accumulation d'acides. Mais ils considéraient généralement les acides comme de simples produits de dégradation incomplète des glucides. La même conception se retrouve chez Molliard (1924) quant à la genèse d'acides organiques dans les cultures de *Sterigmatocystis nigra*.

Enfin, Somers (1951) a montré que des inhibiteurs des oxydations tels que l'azoture, l'iodoacétate de Na, entravent l'accumulation d'acides par les feuilles de *Bryophyllum Daigremontianum*. Ces inhibiteurs ainsi que le cyanure de K, inhibent également leur dégradation, d'après Somers.

J'ai entrepris d'abord l'examen du bilan des oxydations et de la fixation de l'anhydride carbonique, effectuées par les feuilles de *Bryophyllum*, dans diffé-

¹ Le terme de «réserve» ici ne postule pas l'accumulation. Il peut s'agir d'une réserve potentielle, d'une faculté de genèse, compte tenu de l'instabilité de l'acide oxalacétique.

rentes conditions d'oxygénation, en présence ou en l'absence de CO_2 , externe.

Cette étude prélude à celle du mécanisme de l'utilisation du carbone de ces acides, dans la photosynthèse.

B. Méthodes & techniques

1. Techniques générales et d'échanges de gaz

Le *Bryophyllum Daigremontianum* Berger, Crassulacée originaire de Madagascar, a été cultivé en serres. Tous les pieds sont issus du même individu et forment donc un clone.

Seules les feuilles n° 3 (le n° 1 étant attribué aux feuilles incomplètement développées, visibles au sommet de la plante) ont été systématiquement utilisées.

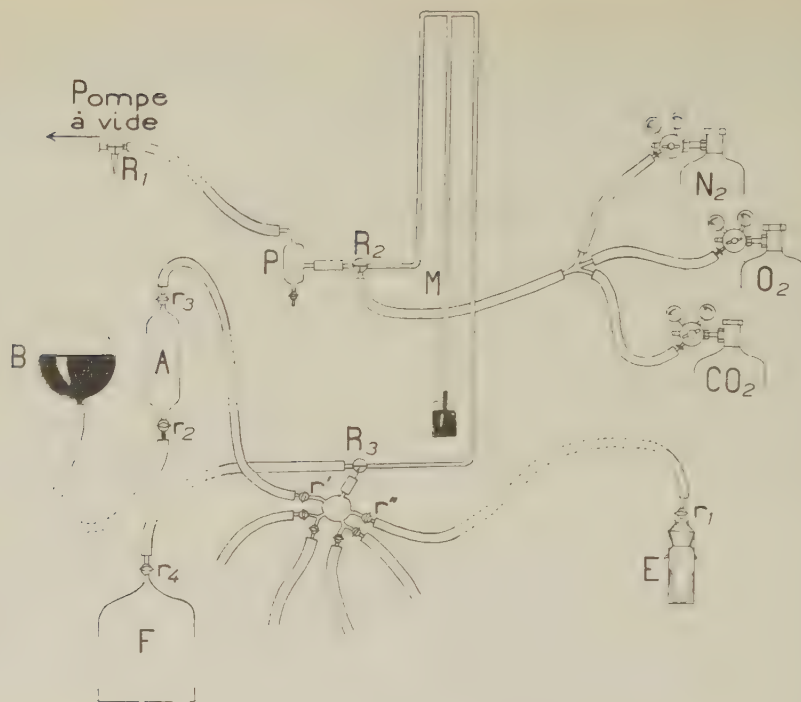
Les feuilles, prélevées à une heure déterminée, sont triées et réparties en lots de 2 (analyses des échanges de gaz) ou de 5 (dosages de l'acidité). Les lots sont pesés et introduits dans des enceintes en verre de 200 cm³ environ (analyses de gaz) ou de 500 cm³ (dosages de l'acidité). Chaque enceinte est munie d'un bouchon rodé portant un robinet. Un fragment de papier buvard imbibé d'eau entretient la saturation de l'atmosphère en vapeur d'eau à l'intérieur de chaque enceinte.

L'atmosphère des enceintes est remplacée par un mélange de CO_2 , O_2 et N_2 , en proportions connues. Les enceintes sont ensuite placées à l'obscurité, à température constante, pendant une période déterminée. Pour les lots destinés aux analyses de gaz, les gaz sont alors extraits, analysés et les feuilles desséchées à $T=100^\circ \text{C}$ jusqu'à poids constant. Les lots destinés à l'analyse de l'acidité sont desséchés rapidement à température inférieure à 80°C dans un courant d'air. Tous les résultats sont exprimés en cm³ de gaz absorbés ou émis par g. de substance sèche ou en milli-équivalents d'acidité par g. de substance sèche.

En raison de la brièveté des durées d'expériences, j'ai considéré que les variations du poids de la substance sèche pouvaient être négligées.

Préparation des atmosphères et analyse des échanges gazeux. — a) La préparation des atmosphères de composition connue en oxygène, azote et anhydride carbonique, est faite à l'aide d'un distributeur manométrique construit sur les indications de L. Plantefol (Fig. 1). La technique est proche de celle décrite par Umbreit et coll. (1951).

Un flacon de 10 l. F, muni d'un robinet, est relié d'une part à une pompe à vide, d'autre part à des bouteilles de gaz comprimés. Sur le trajet se trouvent un manomètre à mercure M et divers robinets : R_1 (robinet pour la rentrée d'air lors de

Figure 1. — *Distributeur manométrique.*

l'arrêt de la pompe). R_2 (communications pompe flacon-bouteilles de gaz). R_3 (communications flacon-bouteilles de gaz, ou flacon-enceintes). Entre la manomètre et la pompe, un piège à mercure P est utile. Les jonctions sont faites avec du caoutchouc à vide garni de piscine aux extrémités. Le vide est fait dans le flacon F et dans les tubes qui se rendent aux bouteilles, jusqu'aux manodétendeurs. L'un des gaz, celui dont la quantité recherchée est la plus grande, est détendu dans le flacon, jusqu'à la pression atmosphérique. Le vide est à nouveau fait. Après ce rinçage, le même gaz est introduit sous une pression partielle convenable, correspondant à la composition désirée. Puis le flacon F étant fermé, le vide est fait à nouveau dans les tubes, et l'espace nuisible qu'ils présentent est rincé avec le deuxième gaz. Le deuxième gaz est ensuite détendu dans les tubes jusqu'à ce qu'il atteigne une pression supérieure à la pression du gaz déjà introduit dans le flacon F. Le robinet r_4 est alors ouvert et la détente du 2ème gaz poursuivie jusqu'à la pression requise. Les mêmes opérations sont faites pour le troisième gaz.

Des abaques permettent aisément la préparation rapide des atmosphères désirées, compte tenu de la pression atmosphérique du moment. Une prise de gaz obtenue par détente de l'atmosphère du flacon F, dans une ampoule à 2 robinets semblable à l'ampoule A, permet le contrôle de la composition de l'atmosphère préparée.

b) Les feuilles sont introduites dans les enceintes telles que E, munies d'un robinet r_1 qui surmonte leur bouchon rodé. Après bouchage, les enceintes sont raccordées aux robinets tels que r. Une ampoule annexe A, reliée à une ampoule B remplie de

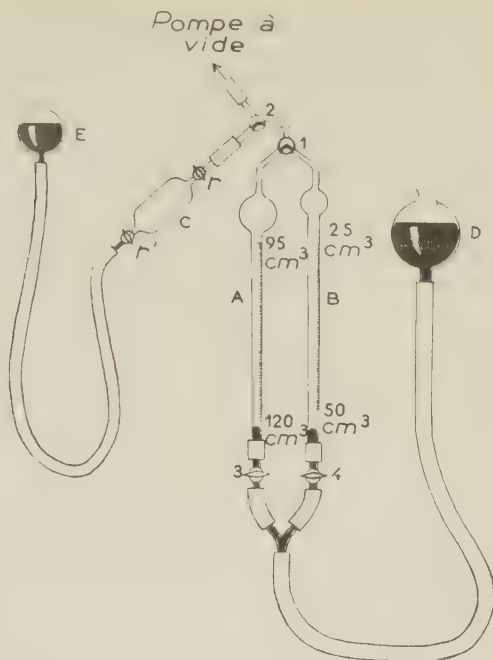


Figure 2. — Mélangeur de gaz.

mercure ou d'eau acidulée est placée dans le circuit. Les robinets r_1 et r_3 étant ouverts, le vide est fait dans E, A et les tubulures annexes. Puis R_2 isolant la pompe à vide, r_4 est ouvert et l'atmosphère de F se détend dans les enceintes et dans l'ampoule A. Le robinet r_1 est fermé et le vide fait à nouveau. Après ce rinçage, une nouvelle détente de l'atmosphère de F permet de remplir de gaz les enceintes, mais à une pression inférieure à la pression atmosphérique. Le robinet R_2 étant fermé, ainsi que r_4 , le gaz de l'ampoule A est refoulé dans les enceintes à l'aide du liquide de B. L'ampoule A sert ainsi de réserve accessoire pour remplir les enceintes jusqu'à ce que la pression atmosphérique soit atteinte.

De nombreuses variantes de manipulation sont possibles. Parmi celles-ci le remplissage direct des enceintes telles que E par détente des gaz des bouteilles. Si cette dernière manière d'opérer peut convenir pour les préparations des atmosphères ne renfermant que de l'oxygène et de l'azote, elle ne convient pas pour l'introduction du CO_2 dont la détente directe, très endothermique, ne permet pas de réaliser une composition homogène des atmosphères des différentes enceintes.

c) Les enceintes sont placées dans une chambre ou une étuve, à température constante, et après la durée choisie, les gaz sont extraits, recueillis dans des ampoules jaugées munies de 2 robinets du type de l'ampoule A et la pression des gaz y est déterminée (voir Plantefol et Moysé, 1948) — Le volume des gaz est ensuite calculé à 0°C et 760 mm Hg, pour comparaison.

d) L'analyse des gaz est faite à l'aide des techniques de Plantefol (1932), le CO_2 étant absorbé par la potasse, l' O_2 par le phosphore blanc.

Lors de l'emploi d'atmosphères renfermant plus de 40 % d' O_2 et, en raison des

risques d'inflammation du phosphore, les atmosphères sont d'abord diluées avec de l'azote purifié, à l'aide du mélangeur décrit ci-dessous (Fig. 2).

Le mélangeur comprend 2 tubes calibrés, gradués, A et B, réunis par un robinet 1 à voie coudée à 120° . Un autre robinet semblable, 2, permet de mettre en communication les tubes gradués tour à tour avec une pompe à vide et avec une ampoule à 2 robinets C, elle-même reliée à une ampoule de manœuvre remplie de mercure E. Les 2 tubes gradués sont reliés par leur base à des robinets 3 et 4 qui permettent de les mettre en communication avec une ampoule de manœuvre D remplie de mercure. Les deux tubes A et B sont remplis de mercure, ce dernier affleurant au-dessus du robinet 1. Une ampoule C, remplie préalablement d'azote est mise en place. Le vide est fait dans l'espace nuisible entre les robinets r et l. L'aspiration étant interrompue, le robinet r est ouvert, puis fermé. Le vide est fait à nouveau entre les robinets r et l. Après un deuxième rinçage, l'ampoule C et le tube A sont mis en communication, l'azote est à la fois aspiré et poussé dans le tube A, à l'aide des ampoules de manœuvre. Son volume est ajusté en A au volume choisi à une pression légèrement supérieure à la pression atmosphérique. L'ampoule C est remplacée par une ampoule similaire contenant le gaz riche en oxygène, à analyser et les mêmes opérations sont faites afin d'introduire ce gaz dans le tube B. Après 5 minutes, les gaz renfermés dans les tubes A et B sont détendus à la pression atmosphérique par la manœuvre des robinets 1 et 2, l'ampoule C ayant été enlevée.

Une ampoule du même type étant fixée et remplie de mercure, le vide est fait dans l'espace nuisible entre les robinets r et l et les gaz renfermés dans les tubes A et B sont tour à tour chassés dans cette ampoule dont le gaz peut être analysé sans risque.

Les causes d'erreur peuvent être réduites par une correction de jauge (volume du trou de la clé du robinet 1) et par une lecture des graduations au viseur (voir Plantefol, 1933).

Le dosage du CO_2 , avant dilution et après dilution, permet de contrôler celle-ci.

Essais préliminaires. —

Plantefol (1938) a signalé que les tissus végétaux pouvaient être très sensibles à l'extraction de leur atmosphère interne par le vide. Audus (1939, 1941) a fait une étude systématique des modifications des échanges gazeux foliaires par les simples manipulations, coupures, frottements et torsions.

Une vérification de l'influence de la technique d'extraction des gaz internes, par le vide, a été faite sur les feuilles de *Bryophyllum* (Tab. 1) Elle montre qu'un séjour de 5 minutes dans le vide n'est pas sans effet sur les échanges gazeux. L'intensité respiratoire est augmentée de 25 pour cent si l'on considère l'absorption d' O_2 . Une émission de CO_2 se manifeste, alors qu'elle est à peine perceptible avec les feuilles témoins, non traitées.

Le passage au vide détermine donc une accélération des oxydations, accélération d'ailleurs temporaire, et même une brève inversion des échanges de CO_2 .

Il est vraisemblable que cette action résulte d'un trouble des échanges entre protoplasme et vacuoles, accompagné d'une oxydation partielle des acides organiques vacuolaires, comparable à celle que l'on peut observer lors de l'étiollement des feuilles (Moyse, 1950).

Ce trouble n'est pas irréversible. L'absorption de CO_2 se rétablit rapidement et les feuilles conservent par ailleurs la faculté de développer des racines et des gemmes lorsqu'elles sont abandonnées sur du coton humide.

Tableau 1. *Echanges gazeux des feuilles de Bryophyllum Daigremontianum Berger, à l'obscurité, après un séjour dans le vide poussé jusqu'à la pression correspondant à la tension de vapeur d'eau, pendant 5 minutes. — Durée de l'expérience: 1 heure. T = 17° C.*

	Feuilles soumises au vide préalable						Feuilles témoins					
	1	2	3	4	5	Moyenne	1	2	3	4	5	Moyenne
O ₂ absorbé cm ³ par h. et g. de substance sèche	0,29	0,36	0,67	0,40	0,57	0,46	0,33	0,34	0,22	0,33	0,38	0,32
CO ₂ émis cm ³ par h. et g. de substance sèche	0,12	0,12	0,36	0,31	0,33	0,25	0	0	0,05	0	0	0,01

2. Dosages d'acidité

Le pH des jus de presse des feuilles est de 5 à 5,4. Le dosage de l'acidité libre a été fait de la manière suivante.

Les feuilles sont desséchées par l'action de la chaleur modérée (80° C, puis 70° C) dans un courant d'air pendant quelques heures, puis en dessiccateur.

Après broyage, les acides sont extraits à 3 reprises successives par l'eau à 80° C. Les liquides sont réunis et l'acidité dosée par NaOH 0,1 N en présence de phénolphtaléine.

Des essais préalables ont été faits afin de comparer cette technique à celle utilisée par Thomas et coll. (extraction directe, par l'eau à 100° C., à partir de tissus frais) et à l'extraction hydroalcoolique après fixation des tissus par l'alcool bouillant, distillation de l'alcool sous pression réduite à 40° C. et reprise par l'eau. (Tab. 2.)

Les différences sont faibles. Des pertes peuvent être attribuées en majeure partie au départ de l'acide oxalique (Somers, 1951).

Tableau 2. *Acidité des feuilles en milli-équivalents par g. de tissu sec (Moyenne de 3 essais).*

a) sur tissus frais, par l'eau à 100° C	b) sur tissus secs, par l'eau à 80° C	c) sur tissus fixés à l'alcool et extraits par l'alcool à 60°
1,42	1,44	1,47

3. Dosages des glucides

A titre de contrôle des analyses de glucides ont été faites sur des feuilles témoins, afin de déterminer les variations glucidiques pendant la durée des expériences.

Les feuilles sont fixées dans l'alcool à 95 . à l'ébullition et, après broyage des tissus, l'extraction des glucides solubles est poursuivie par l'alcool à 60 .

L'alcool est éliminé par distillation sous pression réduite. Les glucides solubles sont repris par l'eau. Une fraction de la liqueur est réservée pour essai d'hydrolyse enzymatique avant défécation. L'autre fraction est déféquée immédiatement par la quantité minimum d'acétate basique de Pb. L'excès de sel de Pb est éliminé par addi-

tion de $\text{PO}_4\text{Na}_2\text{H}$. Après filtration, cette fraction est utilisée pour le dosage des glucides réducteurs libres seulement.

Les déterminations suivantes sont faites sur des parties aliquotes des solutions, par la méthode de Bertrand:

- a) réducteurs libres,
- b) réducteurs après hydrolyse à l'invertase,
- c) réducteurs restant, après hydrolyse à l'invertase et action de SO_4H_2 0,35 N, à l'ébullition, pendant $\frac{1}{2}$ heure,
L'action de l'acide détruit dans ces conditions la majeure partie du sédoheptose. (Wolf, 1937; Pucher et coll., 1947 a)
- d) réducteurs obtenus après reprise aqueuse du résidu d'extraction et action de HCl à 4 pour cent, à l'ébullition, pendant 3 h. Le résultat obtenu est proportionnel à la teneur des tissus en amidon.
La différence des résultats b)—a) peut être rapportée au saccharose et la différence b)—c) est proportionnelle aux teneurs des tissus en sédoheptose.

C. Expériences et résultats

I — L'absorption d'O₂ et l'acidité des feuilles à l'obscurité, en fonction de la tension partielle d'O₂ dans l'atmosphère

Cette première série d'expériences, faite en 1951, comprend l'analyse des échanges gazeux de feuilles n°3 et n°2 prélevées le soir à un moment où, appauvries en acides organiques, elles entrent dans une période de carboxylation prédominante. Elles sont mises à l'obscurité en présence des atmosphères d'azote et d'oxygène de compositions suivantes: — Azote purifié ($\text{O}_2 < 0,1\%$) mélanges d'oxygène et d'azote, O_2 présentant en volume les proportions: 1, 2, 5, 10, 21 (correspondant sensiblement à l'air ordinaire), 40, 60, 80 pour cent, et oxygène purifié.

Ces expériences ont été faites pendant des durées longues (14 à 16 h.), à température constante (17°C).

Le tableau 3 présente les résultats, moyennes de 2 analyses de gaz faites dans chaque cas sur 2 lots semblablement traités.

Les dosages d'acidité libre n'ont été faits qu'avec les feuilles n°3.

Les résultats transcrits sont les moyennes de 2 dosages.

Les gains d'acidité théoriques, calculés d'après les réactions (1) (2) et (3), résumées sous la forme de la réaction (4) ci-après, sont indiqués dans la colonne d. Il est admis alors que tout les produits d'oxydation des glucides sont engagés dans la β -carboxylation, l'absorption d'une molécule-gramme d' O_2 permettant la formation de 2 équivalents d'acidité.

Les réactions caractéristiques des oxydations respiratoires et de la β -carboxylation peuvent être schématisées ainsi:

Tableau 3.

Exp. I. 8 Oct. 1951				Exp. II. 22 Oct. 1951		
Feuilles n° 3, de 10 ^{cm} ± 0,5 ^{cm} de long (prélevées entre 18 et 19 h.)				Feuilles n° 2, de 8 ^{cm} ± 0,5 ^{cm} de long (prélevées entre 18 et 19 h.)		
Obscurité, durée: 14 h. 30 ± 0 h. 30				Obscurité, durée: 15 h. 45 ± 0 h. 30		
T = 17° C, (CO ₂) initiale = 0				T = 17° C, (CO ₂) initiale = 0		
cm ³ d'O ₂ absorbé) par g. de substance cm ³ de CO ₂ émis) sèche et par h.						
gains d'acidité libre en m. éq. par g. de substance sèche, et par h.						
Atmosphères initiales	a cm ³ O ₂ absorbé	b cm ³ CO ₂ émis	c gains d'acidité	d gains d'acidité calculés en fonc- tion de O ₂ fixé	a' cm ³ O ₂ absorbé	b' cm ³ CO ₂ émis
N ₂ (O ₂ < 0,1 %)	< 0,01	0	0,026	—	< 0,01	0
O ₂ = 1 %.....	0,13	0	0,045	0,010	0,14	0
O ₂ = 2 %.....	0,18	0	0,048	0,015	0,21	0
O ₂ = 5 %.....	0,25	0	0,045	0,021	0,24	traces
O ₂ = 10 %.....	0,28	traces	0,046	0,024	0,20	0,03
O ₂ = 21 %.....	0,48	0	0,049	0,041	0,33	0,01
O ₂ = 40 %.....	0,67	traces	0,045	0,057	0,59	0,02
O ₂ = 60 %.....	0,72	0	0,041	0,061	0,57	0,02
O ₂ = 80 %.....	—	—	—	—	0,57	traces
O ₂ = 100 %.....	0,88	0,05	0,040	0,075	0,55	0,05

Acidité initiale des témoins | 0,55 à 0,60 m. éq. par g. de substance sèche

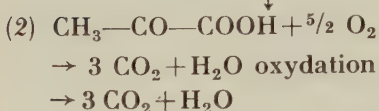
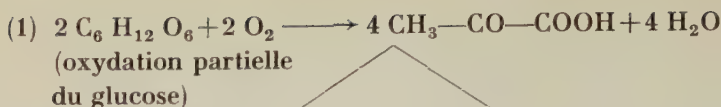
Teneur en eau | 1440 ± 80 pour 100 de substance sèche | 1330 ± 70 pour 100 de substance sèche

Souvent, la teneur en eau paraît plus élevée pour les feuilles mises en présence des atmosphères fortement oxygénées. Mais le calcul de la différence des moyennes et de la déviation standard ne permet pas de considérer ces variations comme significatives.

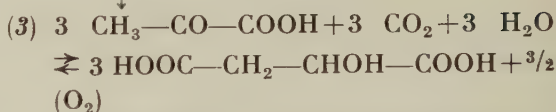
Tableau 4. Contrôle des teneurs en glucides. — Ces contrôles sont faits avec des feuilles n°3 prélevées au début de l'expérience. Une fraction est immédiatement fixée à l'alcool. L'autre est laissée à l'obscurité, à l'air libre, sur du coton humide. Elle est fixée 15 h. après, à la fin de l'expérience. Expérience I. 8 Oct. 1951 — Feuilles n°3 prélevées entre 18 et 19 h. Obscurité, durée 15 h. T = 19°C. Glucides exprimés en mg. de glucose par g. de substance sèche.

	a) Réducteur libre	b) Réd. après hydrolyse à l'invertase	c) Réd. après action de SO ₄ H ₂ 0,35 N	b) — a) Saccharose	b) — c) Sédo- heptose	d) Amidon	Glucides totaux
Début de l'Exp.	2,9	6,8	4,0	3,9	2,8	—	—
Fin de l'Exp.	5,9	10,7	8,8	4,8	1,9	19,7	30,4

En fin d'expérience, l'amidon est encore abondant, les feuilles ne sont donc pas carencées en glucides. On observe un accroissement des glucides solubles, qui peut être rapporté aux glucides réducteurs libres différents du sédoheptose.

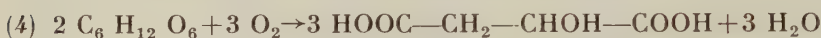


oxydation totale d'une
 fraction de l'acide pyruvique formé au cours de
 la réaction (1) (Cycle de
 Krebs)



β-carboxylation et réduction d'une frac-
 tion de l'acide pyruvique formé au cours
 de la réaction (1), le CO₂ provenant de
 la réaction (2). L'acide malique s'accu-
 mule.¹

Ces 3 réactions sont groupées dans la suivante :



Seul le CO₂ provenant des oxydations respiratoires intervient dans la β-carboxylation. Trois O₂ seulement sont nécessaires pour l'accomplissement de la réaction totale, alors que l'oxydation complète de 2 mol. de glucose exige 12 mol. d'O₂. La fixation de 3 O₂ entraîne un gain d'acidité de 6 équivalents.

Discussion. — Les observations suivantes peuvent être faites :

1. — La fixation d'O₂, caractéristique des oxydations respiratoires, augmente avec la tension partielle d'O₂ selon une courbe qui est une hyperbole (Moise, 1951). Elle a même allure que celle constatée dans d'autres cas, en particulier avec les germinations de Blé, d'Orge, de Riz (Taylor, 1942; Vlamis et coll., 1943), les racines d'Oignon (Berry et coll., 1949). Le maximum n'est atteint qu'aux tensions supérieures à la pression partielle de l'O₂ dans l'air ordinaire. Ce fait tend à montrer incidemment que, quelle que soit la tension partielle d'O₂, sa vitesse de diffusion dans les tissus, qui est liée à sa tension dans l'atmosphère, limite bien la vitesse des oxydations (Berry et coll., 1949).

2. — Le dégagement de CO₂ est nul aux basses tensions d'O₂ (carboxylation dominante). Il n'apparaît systématiquement qu'en présence des fortes tensions d'O₂. A ces fortes tensions, la carboxylation est insuffisante pour retenir tout le CO₂ produit par les oxydations respiratoires pendant la durée de l'expérience.

3. — Le quotient respiratoire apparent, $Q. R. = \frac{\text{vol. CO}_2 \text{ émis}}{\text{vol. CO}_2 \text{ absorbé}}$, est nul

¹ Les variations quantitatives des acides organiques : malique, citrique, isocitrique, succinique, seront décrites ultérieurement. Il a été vérifié que la teneur en acide malique était la principale variable. Il n'y a pas libération d'O₂, la formule n'exprime qu'un bilan.

pour les basses tensions. Aux fortes pressions partielles d'O₂, ses valeurs sont encore très faibles (0,05 à 0,1).

4. — Les variations de l'acidité des feuilles montrent qu'aux basses tensions d'O₂ le gain d'abord faible, dans l'atmosphère d'azote, croît avec la tension d'O₂, atteint son maximum pour des tensions comprises entre 5 et 21 pour cent et décroît ensuite légèrement.

En anaérobiose, l'augmentation de l'acidité, si elle est attribuable à une élévation de la teneur des tissus en acide malique, peut être liée à une réaction fermentaire. Cette réaction fermentaire devrait fournir à la fois l'acide pyruvique et le CO₂ nécessaires à la genèse de l'acide malique par le mécanisme de β -carboxylation.

Si la carboxylation n'intervenait pas, on constaterait une émission de CO₂, ce qui n'est pas le cas.

Le faible gain d'acidité, dans l'azote, montre que cette réaction est limitée. Son inhibition partielle serait vraisemblablement liée à l'intoxication des cellules par les produits de fermentation comparables à ceux dont la formation a été observée en présence de très fortes tensions de CO₂ (Thomas et coll., 1954). La mort rapide des feuilles, vers la vingtième heure de séjour dans l'azote, tendrait à confirmer cette interprétation.

En présence des faibles tensions d'O₂ (de 1 à 10 pour cent), les oxydations, y compris celles de décarboxylation, se déroulent à une vitesse suffisamment faible pour que tout le CO₂ émis (réaction 2 ou réaction 3 dans le sens de la décarboxylation) puisse être repris, ou conservé, sous forme carboxylée.

Si l'on compare les gains d'acidité réels, mesurés, et les gains possibles, calculés d'après la fixation d'O₂, (colonne *d*), on constate que les premiers sont plus élevés que ne le laisseraient prévoir les seconds. Ce fait peut s'expliquer par l'intervention des oxydo-réductions fermentaires, accompagnées d'une genèse d'acides sans absorption correspondante d'O₂.

A la tension d'O₂ de 20 pour cent, les gains réels d'acidité et les gains calculés sont très voisins, le maximum de gain d'acidité est réalisé dans les conditions de la durée expérimentale.

Avec les fortes tensions d'O₂, le renversement de la réaction de carboxylation se manifeste, du CO₂ est émis. La carboxylation n'assure plus sa fixation, l'acidité diminue et ses gains sont nettement plus faibles que ceux qui peuvent être calculés d'après l'absorption d'O₂. Ces faits confirment les variations observées par Pucher et coll. (1947 c) et Thomas et coll. (1949), dans l'acidité des feuilles maintenues à l'obscurité pendant de longues durées dans l'air ordinaire.

La différence que l'on constate avec les fortes tensions réside dans le raccourcissement de la période de carboxylation dominante.

II — L'absorption de CO_2 externe a l'obscurité et l'acidité des feuilles, en fonction de la tension partielle d' O_2 dans l'atmosphère

Une seconde série d'expériences, faite en 1952, permet de compléter ces résultats et de préciser les conditions d'oxygénation nécessaires à la fixation du CO_2 externe.

Les feuilles utilisées sont identiques aux précédentes. Les conditions d'expérience sont les mêmes, aux tensions de CO_2 externe et aux durées près.

Les atmosphères utilisées renferment 7 % de CO_2 et des pressions partielles d' O_2 échelonnées :

— azote ($0_2 < 0,2$ pour cent), 1, 2, 5, 10, 20, 50 et 93 pour cent d' O_2 . Dans chaque cas, la teneur initiale exacte des atmosphères en CO_2 et O_2 est contrôlée.

Les différences entre les différentes teneurs en CO_2 n'excèdent pas $\pm 0,2$ du pourcentage total.

Les durées sont de 4, 10, 14 et 20 heures, sauf pour les feuilles en anaérobiose, qui présentent des signes précurseurs de la mort entre 14 et 20 h. (perte de turgescence, assombrissement de la pigmentation).

Comme dans la série précédente, les feuilles sont prélevées le soir, alors qu'elles sont appauvries en acides. J'ai négligé les variations de poids de substance sèche au cours du temps, en raison de la brièveté des durées d'expériences. Les résultats sont exprimés de la même manière que précédemment.

1. — La fixation de CO_2 . — Le tableau 5 résume les résultats des analyses d'échanges de gaz, après séjour des feuilles en présence de toute la gamme des tensions utilisées.

Tableau 5. Exp. III. 30 Juin et 3 Juillet 1952. Feuilles n°3, de $10 \text{ cm} \pm 0,5 \text{ cm}$ de long, prélevées entre 18 et 19 h. Obscurité, $T = 19^\circ \text{ C}$. (CO_2) initiale = $7 \pm 0,2$ p. cent. Cm^3 de CO_2 et d' O_2 absorbé par g. de substance sèche.

Atmosphères initiales	CO_2 absorbé				O_2 absorbé			
	0-4 h.	0-10 h.	0-14 h.	0-20 h.	0-4 h.	0-10 h.	0-14 h.	0-20 h.
N_2 ($\text{O}_2 < 0,2 \%$)	0	0,7	0,3	—	0,2	0,3	0,3	—
$\text{O}_2 = 1 \%$...	0,6	10,4	10,7	4,1	1,1	2,3	2,5	2,3
$\text{O}_2 = 2 \%$...	0,9	11,5	14,4	3,8	1,5	3,2	4,1	4,1
$\text{O}_2 = 5 \%$...	1,7	12,3	14,9	4,2	1,4	4,0	4,2	4,8
$\text{O}_2 = 10 \%$...	1,6	14,6	15,8	7,4	1,5	3,9	5,1	5,4
$\text{O}_2 = 20 \%$...	2,5	17,2	11,8	6,8	1,5	4,5	5,9	7,1
$\text{O}_2 = 50 \%$...	3,4	15,5	10,9	5,5	2,9	5,9	9,6	10,4
$\text{O}_2 = 93 \%$...	3,2	15,1	11,2	4,1	3,2	7,4	11,0	13,2

Teneur en eau: 1680 ± 80 pour cent de substance sèche.

Tableau 6. Expérience III — 30 Juin 1952. *Contrôle des teneurs en glucides.* — Ces contrôles sont faits dans les mêmes conditions que les précédents avec des feuilles qui sont restées à l'obscurité, à l'air libre, pendant 15 et 21 h. Glucides exprimés en mg. de glucose par g. de substance sèche.

	a) Réducteur libre	b) Réd. après hydrolyse à l'invertase	c) Réd. après action de SO ₄ H ₂ 0,35 N	b)—a) Saccharose	b)—c) Sédo- heptose	d) Amidon	Glucides totaux
Début de l'Exp.	4,4	13,9	11,5	9,5	2,0	38,4	52,3
Après 15 h. ...	6,9	16,1	13,1	9,2	3,0	20,7	36,8
Après 21 h. fin de l'Exp.	7,6	18,5	16,8	10,9	1,7	15,5	34,0

Les mêmes remarques peuvent être faites que lors de l'expérience I, les feuilles ne sont pas carencées en glucides en fin d'expérience; la teneur en amidon a décru, la teneur en glucides réducteurs autres que le sédoheptose a augmenté.

La tension partielle de CO₂, dans les atmosphères, en fin d'expérience, est naturellement toujours plus faible qu'au début. Dans l'atmosphère d'azote, elle a diminué de 7,2 à 7. Après 4 h., elle est de 3,2 dans l'atmosphère à 1 p. cent d'O₂, et de 1,7 dans l'atmosphère à 20 p. cent d'O₂. C'est la valeur la plus faible pour cette durée. Après 10 h. elle est de 3,3 pour l'atmosphère à 1 p. cent d'O₂ et de 0,8 pour l'atmosphère à 20 p. cent d'O₂. Cette dernière valeur est la plus faible de toute la série.

Pour les atmosphères plus riches en O₂, la valeur de la tension de CO₂ s'est maintenue entre 2,1 (O₂=50 p. cent, 14 h.) et 5,8 (O₂=50 p. cent, 4 h.).

Les feuilles ont toujours eu un excès de CO₂ à leur disposition, par rapport au CO₂ libéré par les oxydations respiratoires.

La tension partielle d'O₂, dans les atmosphères, en fin d'expérience, est également toujours plus faible qu'au début. Dans l'atmosphère d'azote, elle est nulle après 10 h. Elle n'est plus que de 0,1 p. cent après 10 h., pour l'atmosphère à 1 p. cent d'O₂, de 0,44 après 14 h. pour l'atmosphère à 2 p. cent d'O₂ et de 3,35 p. cent pour l'atmosphère à 5 % d'O₂ après 20 h.

Il en est tenu compte dans l'interprétation des résultats.

Pour les atmosphères plus riches en O₂, les diminutions par rapport à la tension initiale, sont toujours plus faibles.

Le tableau 7 présente les résultats d'une autre série (Exp. V) faite en utilisant seulement les tensions élevées d'O₂, quelques mois après l'Exp. III.

Le tableau 8 indique les résultats d'une expérience témoin (Exp. IV), faite quelques jours après l'expérience III, afin de déterminer la fixation d'O₂ par des feuilles comparables, développées à la même saison, pendant la durée de carboxylation intense et dominante (10 h.), en l'absence de CO₂ externe.

Tableau 7. Exp. V — 22 Septembre 1952. Feuilles n°3, de 10 cm \pm 0,5 cm de long, prélevées entre 18 et 19 h. Obscurité, T=19° C. (CO₂) initiale=6,8 \pm 0,1 p. cent. Cm³ de CO₂ et d'O₂ absorbé par g. de substance sèche.

Atmosphères initiales	CO ₂ absorbé				O ₂ absorbé			
	0—4 h.	0—10 h.	0—14 h.	0—20 h.	0—4 h.	0—10 h.	0—14 h.	0—20 h.
O ₂ = 20 % ...	1,7	10,3	9,9	8,6	0,8	3,1	3,6	4,3
O ₂ = 50 % ...	2,4	8,2	9,1	6,8	2,6	4,2	6,4	8,0
O ₂ = 93,2 % ...	2,2	9,3	9,6	5,9	2,5	5,1	6,8	9,4

Teneur en eau: 1170 \pm 40 pour cent de substance sèche.
La tension partielle de CO₂ n'est jamais descendue au-dessous de 2 pour cent.

Tableau 8. Exp. Témoin IV — 10 Juillet 1952. Feuilles n°3, de 10 cm \pm 0,5 cm de long, prélevées entre 18 et 19 h. Obscurité, T=19° C. Atmosphères sans CO₂. Durée 10 h. Cm³ d'O₂ absorbé par g. de substance sèche.

Atmosphères initiales	N ₂ (O ₂ < 0,2 %)	O ₂ = 1 %	O ₂ = 2 %	O ₂ = 5 %	O ₂ = 10 %	O ₂ = 20 %	O ₂ = 50 %	O ₂ = 100 %
O ₂ absorbé ...	0,2	2,1	3,0	4,2	4,6	5,3	8,4	9,1

Pendant la durée de l'expérience, aucune trace de CO₂ n'a été trouvée, quelle que soit la tension d'O₂ utilisée.

Discussion. — Les résultats des Exp. III et V confirment ceux obtenus par Thomas et coll. (1949, 1954) à propos de la prédominance de la carboxylation pendant une dizaine d'heures, dans l'air ordinaire enrichi en CO₂, avec, après cette période, renversement du phénomène et émission de CO₂. L'émission de CO₂, après 10 h., se traduit par une diminution apparente de la quantité de CO₂ fixée.

De plus, on peut faire les constatations suivantes :

1. — En anaérobiose, la fixation du CO₂ externe est pratiquement nulle, ainsi que je l'ai décrit précédemment (Moise, 1953).

2. — La fixation de CO₂ externe exige la présence d'O₂ et l'allure du phénomène au cours du temps est la même quelle que soit la tension d'O₂ dans l'atmosphère : d'abord fixation (carboxylation dominante) suivie d'émission (décarboxylation dominante).

3. — Si l'on examine chaque durée particulière, les faits suivants se manifestent :

a) Les maximums de fixation sont voisins (11 à 15 cm³ de CO₂ par g. de substance sèche, quelle que soit la tension d'O₂ externe. La quantité la plus

élevée a été obtenue après 10 h. dans l'atmosphère à 20 pour cent d'O₂ (17 cm³, 2 de CO₂ fixé).

Il existe vraisemblablement un maximum de carboxylation, lié à l'abondance des réserves glucidiques et à la cinétique de la réaction réversible de fixation de CO₂.

b) Plus la tension initiale d'O₂ est forte, plus est rapide la fixation de CO₂ (durées de 4 h.). La vitesse de la fixation croît donc avec la tension d'O₂, comme croît la vitesse des oxydations caractérisée par la rapidité de l'absorption d'O₂.

c) Réciproquement, plus l'optimum de fixation est rapidement atteint, plus est précoce l'émission de CO₂ qui lui fait suite.

d) Lorsque les tensions partielles d'O₂ se maintiennent à des valeurs assez élevées (à partir de 10 pour cent au début), la vitesse de la décarboxylation est d'autant plus rapide que la tension d'O₂ est grande.

Remarque: La durée même des expériences entraîne des modifications des conditions initiales. Aux faibles tensions partielles d'O₂, l'affaiblissement rapide des quantités d'oxygène résiduel appelle une précision. Après 10 h., les feuilles mises initialement en présence d'une atmosphère oxygénée à 1 pour cent sont soumises à l'anaérobiose presque complète, condition de la série N.

Après 14 h. les feuilles mises en présence initialement d'une atmosphère oxygénée à 2 pour cent, se trouvent dans des conditions intermédiaires entre celles qui étaient soumises à des teneurs en oxygène de 2 et de 1 pour cent. Enfin, entre 14 h. et 20 h., les feuilles initialement placées dans une atmosphère renfermant 5 pour cent d'O₂ n'en disposent guère plus que celles mises initialement en présence d'O₂ à 2 pour cent.

Il en résulte un affaiblissement du maximum de fixation, du maximum de carboxylation, qui sont atteints plus tardivement.

Il existe donc un lien direct entre la vitesse de la carboxylation et celle des oxydations respiratoires, de même qu'entre la vitesse de décarboxylation et celle des oxydations.

4. — Si l'on compare les fixations d'O₂ en 10 h., faites par des feuilles prélevées à la même époque, en présence de CO₂ externe (Tab. 5, Exp. III) et en l'absence de CO₂ externe (Tab. 8, Exp. Témoin IV), on constate que la présence de CO₂ retarde l'absorption d'O₂. Si l'on se reporte au schéma de la p. 464, ce fait peut s'interpréter comme la conséquence d'une dérivation de l'acide pyruvique provenant de l'oxydation des glucides vers la carboxylation, et un amoindrissement de l'activité du cycle de Krebs. L'oxygène absorbé serait alors utilisé surtout dans l'oxydation partielle des glucides, avec genèse d'acide pyruvique et ce dernier, plus engagé dans la voie de la carboxylation, se trouverait en partie au moins soustrait à la poursuite des oxydations génératrices de CO₂, opérées normalement au cours du cycle tricarboxylique, avec participation d'O₂.

Tableau 9. Exp. III. Gains d'acidité en m. éq. par g. de substance sèche. (CO_2) initiale = $7 \pm 0,2$ p. cent.

Atmosphères initiales	0—4 h.	0—10 h.	0—14 h.	0—20 h.
N_2 ($\text{O}_2 < 0,2$ %).....	0,62	0,56	0,45	
$\text{O}_2 = 1$ %	0,63	1,20	1,09	1,03
$\text{O}_2 = 2$ %	0,60	0,99	1,19	1,12
$\text{O}_2 = 5$ %	0,62	1,07	1,22	1,15
$\text{O}_2 = 10$ %	0,66	1,22	1,20	1,19
$\text{O}_2 = 20$ %	0,74	1,31	1,28	1,14
$\text{O}_2 = 50$ %	0,79	1,30	1,11	0,94
$\text{O}_2 = 93$ %	0,82	1,47	1,13	0,90
TEMOIN air ordinaire (moyenne de 2 mesures)	0,36	1,12	1,02	1,05

2. — *Les variations de l'acidité.* — Le tableau 9 indique les gains d'acidité observés au cours de l'expérience III, pour des feuilles semblablement traitées.

Les témoins initiaux renferment 0,41 m. éq. d'acides libres par g. de substance sèche.

Discussion. Les gains d'acidité observés s'accordent avec les résultats obtenus sur la fixation de CO_2 , confirment les résultats de Thomas et coll. et de plus, mettent en évidence les faits suivants :

1. — En anaérobiose, malgré l'absence de fixation de CO_2 externe, les gains d'acidité sont nets (cf. Exp. I), mais faibles et la durée augmentant, la désacidification est pratiquement nulle.

2. — Les gains d'acidité sont d'autant plus rapides et d'autant plus élevés que la vitesse des oxydations est plus grande (maximum en 4 h. comme en 10 h. pour $\text{O}_2 = 93$ pour cent).

Le maximum d'acidité ne coïncide pas avec le maximum de fixation de CO_2 externe ($\text{O}_2 = 20$ pour cent, 10 h.) car il faut tenir compte également de la reprise du CO_2 libéré par les oxydations respiratoires, or ces oxydations sont d'autant plus intenses que l'atmosphère est plus richement oxygénée.

3. — Lorsque le maximum de gain d'acidité est atteint, les variations ultérieures sont faibles. Aux basses et aux moyennes tensions d' O_2 le taux d'acidité se maintient constant. Par contre, en présence des fortes tensions d' O_2 , la désacidification se manifeste avec netteté, la perte d'acidité étant d'autant plus accentuée que l'oxygénation est plus forte.

4. — Il y a une différence sensible, quoique faible, lorsque la tension d' O_2 est voisine de 20 pour cent, entre les feuilles mises en présence d'une atmosphère riche en CO_2 et les feuilles laissée à l'air libre. Les maximums (10 h.) sont alors un peu plus élevés dans le premier cas que dans le second. Mais

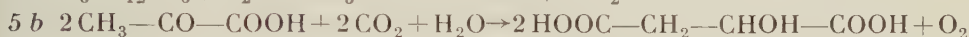
surtout, la vitesse de carboxylation en 4 h. est plus que doublée en présence de CO_2 .

5. — Il est possible, ainsi que l'ont fait Thomas et coll., de calculer quels sont les gains d'acidité théoriques, d'après les quantités de CO_2 fixé, afin de les comparer aux gains d'acidité mesurés.

Le tableau 10 indique les gains d'acidité théoriques calculés, pendant la période de carboxylation dominante, d'après les quantités d' O_2 absorbé (gains correspondants à la reprise du CO_2 libéré par les oxydations respiratoires : réaction 4 précédemment indiquée) et d'après la réaction 5 résumant la fixation du CO_2 externe et la genèse de l'acide malique à partir d'un glucide :



Cette réaction se décompose schématiquement ainsi :



La fixation de 2 mol. grammes de CO_2 entraîne un gain d'acidité de 4 équivalents.

Les bases de ces calculs ne sont évidemment pas rigoureuses.

Les causes d'erreurs sont multiples. On peut signaler les suivantes :

a) La solubilité de CO_2 dans les liquides cellulaires en est une. Si les jus de presse sont acides et ne retiennent que peu de CO_2 , d'après les coefficients de distribution d'Ostwald, il n'en est pas nécessairement de même du protoplasme vivant, qui lui est neutre, sinon alcalin.

L'extraction des gaz par le vide a l'avantage de réduire considérablement la fixation du CO_2 par simple solubilité dans les liquides cellulaires.

b) Une fraction des acides organiques est salifiée. Cette fraction est variable et il n'est pas tenu compte de ses variations qui seront relatées ultérieurement.

c) Les analyses en cours montrent également que la quantité d'acide malique n'est pas la seule variable et que du CO_2 peut être fixé par d'autres voies (cf. Ranson, Addendum in Thomas et coll., 1954).

Quoi qu'il en soit, la comparaison demeure tout de même intéressante.

La comparaison des chiffres des tableaux 9 et 10 montre que pendant la première période de 4 h, les gains d'acidité calculés d'après les échanges de gaz sont plus faibles que les gains mesurés. Ils sont surtout beaucoup plus faibles aux basses tensions d' O_2 , ce qui peut s'expliquer par l'intervention d'une reprise de CO_2 d'origine fermentaire, par un accepteur du type de l'acide pyruvique, lui-même produit par fermentation.

Pour les tensions d' O_2 supérieures à 20 pour cent, la différence est moins explicable, l'intervention de phénomènes fermentaires est peu probable, malgré la présence de 7 pour cent de CO_2 .

Tableau 10. Gains d'acidité calculés en milli-équivalents par g. de substance sèche, d'après l'absorption de CO_2 et d' O_2 pendant la période de carboxylation dominante (données du tableau 5, CO_2 initial = $7 \pm 0,2$ p. cent).

Atmosphères initiales	Période de 0—4 h.			Période de 0—10 h.		
	D'après CO_2 fixé, ne tenant pas compte des oxydations respiratoires	D'après O_2 fixé, ne tenant pas compte de la fixation de CO_2 externe	Total	D'après CO_2 fixé, ne tenant pas compte des oxydations respiratoires	D'après O_2 fixé, ne tenant pas compte de la fixation de CO_2 externe	Total
$\text{O}_2 = 1 \%$	0,05	0,10	0,15	0,92	0,20	1,12
$\text{O}_2 = 2 \%$	0,08	0,13	0,21	1,02	0,28	1,30
$\text{O}_2 = 5 \%$	0,15	0,12	0,27	1,10	0,36	1,46
$\text{O}_2 = 10 \%$	0,14	0,13	0,27	1,30	0,34	1,64
$\text{O}_2 = 20 \%$	0,22	0,13	0,35	1,54	0,40	1,94
$\text{O}_2 = 50 \%$	0,30	0,26	0,56	1,38	0,52	1,90
$\text{O}_2 = 93 \%$	0,28	0,28	0,56	1,34	0,66	2,00

Dans tous les cas, et indépendamment des tensions d' O_2 , les lésions initiales des feuilles, inhérentes à la méthode, ne permettent pas une interprétation poussée des résultats, la durée de 4 h. étant relativement courte. Avec les périodes de 10 h., la comparaison est plus aisée; les disparités sont relativement moins grandes. Si les gains calculés sont généralement un peu plus élevés que les gains mesurés, aux basses tensions, les phénomènes fermentaires paraissent prendre moins d'importance relative par rapport aux échanges de CO_2 et d' O_2 . La concordance entre le calcul et la mesure est assez bonne.

Aux fortes tensions d' O_2 , les gains calculés sont plus élevés, mais ainsi que l'indique la colonne 0—14 h. du tableau 9, les diminutions d'acidité sont proches de se manifester, si elles ne l'ont déjà fait, et l'absorption d' O_2 ne s'accompagne plus alors d'un enrichissement en acides puisqu'au contraire elles précipite la décarboxylation.

3. — *Les quotients des échanges de gaz et les variations de l'acidité.* — Les valeurs des quotients d'échanges $\frac{\text{CO}_2}{\text{O}_2}$ en volumes sont négatifs pendant la phase de carboxylation dominante, si on les compare aux quotients respiratoires habituels, puisqu'il y a absorption et non émission de CO_2 (cf. Thomas et coll., 1949).

Ils sont positifs et leur valeur absolue peut être fort élevée pendant la période de décarboxylation dominante.

Le tableau 11 indique leur répartition, le calcul en ayant été fait pour les différentes périodes comprises entre les diverses durées d'expérience, et non sur les durées totales. Sauf pour la durée 0—4 h., le calcul est donc fait d'après les échanges de lots de feuilles différents, aussi je ne crois pas pouvoir tenir compte d'une manière précise des résultats en valeur absolue.

Tableau 11. *Signes des Quotients d'échanges* $\frac{\text{CO}_2}{\text{O}_2}$.

Atmosphères initiales	0—4 h.	4 h.—10 h.	10 h.—14 h.	14 h.—20 h.
O ₂ = 1 %	—	—	—	+
O ₂ = 2 %	—	—	—	indéterminé
O ₂ = 5 %	—	—	—	++
O ₂ = 10 %	—	—	—	++
O ₂ = 20 %	—	—	++	++
O ₂ = 50 %	—	—	+	++
O ₂ = 93 %	—	—	+	++

Le signe — désigne les quotients de carboxylation <1 en valeur absolue, le signe — les mêmes quotients négatifs >1 en valeur absolue, le signe + les quotients compris entre 1 et 2, le signe ++ les quotients supérieurs à 2.

De 0 à 4 h., le quotient $\frac{\text{CO}_2}{\text{O}_2}$ est toujours compris entre $-0,5$ et $-1,7$. En présence des basses tensions d'O₂, il est plus faible en valeur absolue, traduisant la faible intensité de la fixation de CO₂ externe par rapport aux oxydo-réductions fermentaires génératrices de CO₂ qui peut être fixé.

Les plus fortes valeurs absolues des quotients négatifs se situent toutes entre 4 h. et 10 h. Elles décroissent lorsqu'augmente la tension d'O₂, de -8 (O₂=1 %) à $-1,6$ (O₂=20 %) et à $-3,1$ (O₂=93 %), traduisant l'affaiblissement de la fixation du CO₂ externe, lorsque le CO₂ libéré par les oxydations est plus abondant. Le quotient atteint la valeur de l'unité (quotient respiratoire glucidique) quand la carboxylation et la décarboxylation s'équilibrent, puis, la tendance à la décarboxylation s'affermir avec la durée, d'autant plus rapidement que la tension d'O₂ est plus élevée, les quotients deviennent alors positifs et rapidement supérieurs à 1, valeur des quotients respiratoires glucidiques.

D. Conclusions

Ces deux séries d'expériences permettent de dégager les conclusions suivantes:

1. — La fixation du CO₂ par β -carboxylation est greffée sur les oxydations cellulaires, le substrat de fixation étant un produit intermédiaire des oxydations.

2. — La fixation concerne aussi bien le CO₂ d'origine respiratoire ou fermentaire que le CO₂ externe, mais en anaérobiose, elle est limitée au premier, vraisemblablement par une inhibition par les produits de fermentation.

3. — La fixation de CO₂ est d'autant plus rapide que les oxydations sont plus intenses. Plus rapidement sont atteints le maximum de fixation et le

maximum d'acidité, plus rapidement intervient également la désacidification par décarboxylation.

4. — La présence de CO_2 externe et sa fixation provoqueraient une déviation de l'acide pyruvique (ou d'une substance voisine), produit intermédiaire des oxydations, vers la β -carboxylation, d'où résulterait un ralentissement de sa propre oxydation.

5. — La décarboxylation à l'obscurité, après une période prolongée, suppose une inversion de l'activité enzymatique dès que le taux maximum d'acidité a été atteint.

Tout se passe comme si l'élévation du taux d'acidité favorisait la décarboxylation dès qu'une concentration maximum d'acide est réalisée. La présence de CO_2 en grandes quantités freine la décarboxylation ou bien favorise la carboxylation compensatrice.

Des études de la cinétique de ces réactions enzymatiques apporteront peut-être quelques éclaircissements sur ce problème.

Résumé

Des feuilles de *Bryophyllum Daigremontianum* Berger sont placées à l'obscurité, dans des atmosphères dont la tension d' O_2 s'échelonne de 0 à 100 pour cent. Ces atmosphères sont, ou bien dépourvues de CO_2 , ou bien en renferment 7 pour cent.

Les analyses des échanges d' O_2 et de CO_2 et celles des variations de l'acidité des tissus, pendant des périodes croissantes, montrent l'influence de la vitesse des oxydations sur la fixation de CO_2 , qu'il s'agisse du CO_2 libéré par ces mêmes oxydations ou du CO_2 présent initialement dans les atmosphères externes.

Ces analyses montrent également le rôle de l' O_2 dans la détermination de la vitesse des oxydations des glucides et des acides organiques.

Des hypothèses sont faites pour rapprocher ces résultats des réactions du métabolisme intermédiaire des oxydations (cycle de Krebs et réactions de β -carboxylation) proposées par les recherches de biochimie.

Bibliographie

- Astruc, A.: Recherches sur l'acidité végétale. — Ann. Sc. Nat. Bot. série 8. 17: 1. 1903.
- Audus, L. J.: Mechanical stimulation and respiration in the green leaf. II — Investigations on a number of angiospermic species. — New Phytol. 38: 284. 1939.
- Mechanical stimulation and respiration in the green leaf. V — The relation of mechanical stimulation effects to leaf turgidity. — New Phytol. 40: 92. 1941.
- Bandurski, R. S. & Greiner, C. M.: The enzymatic synthesis of oxalacetate from phosphoryl-enolpyruvate and carbon dioxide. — Journ. biol. Chem. 204: 781. 1953.

- Bennet-Clark, T. A.: The rôle of the organic acids in plant metabolism. — *New Phytol.* 32: 37. 1933 a, 128, 1933 b.
- Berry, L. J. & Norris, W. E. Jr.: Studies on onion root respiration. I — Velocity of oxygen consumption in different segments of root at different temperatures as a function of partial pressure of oxygen. — *Biochem. Biophys. Acta* 3: 593. 1949.
- Bonner, W. & Bonner, J.: The role of carbon dioxide in acid formation by succulent plants. — *Amer. Journ. Bot.* 35: 113. 1948.
- Ceithalm, J. & Vennesland, B.: The synthesis of tricarboxylic acids by carbon dioxide fixation in parsley root preparations. — *Journ. biol. Chem.* 178: 133. 1949.
- Clendenning, K. A., Waygood, E. R. & Weinberger, P.: The carboxylases of leaves and their role in photosynthesis. — *Canad. Journ. Bot.* 30: 395. 1952.
- Conn, E., Vennesland, B. & Kraemer, L. M.: Distribution of a Triphosphopyridine nucleotide. Specific enzyme catalyzing the reversible oxidative decarboxylation of malic acid in higher plants. — *Arch. Biochem.* 23: 179. 1949.
- Kraus, G.: Ueber die Wasservertheilung in der Pflanze. IV. Die Acidität des Zellsaftes. — *Abhandl. naturf. Ges. zu Hallen.* 16: 141. 1883.
- Molliard, M.: Nouvelles recherches sur la formation d'acides organiques par le *Sterigmatocystis nigra* en milieux déséquilibrés. — *C. R. Acad. Sc., Paris.* 178: 41. 1924.
- Moyse, A.: Respiration et métabolisme azoté (Etude de physiologie foliaire). Hermann et Cie ed. Paris, 1950.
- Les échanges gazeux respiratoires des feuilles de *Bryophyllum Daigremontianum* Berger, en présence d'atmosphères aux pressions d'oxygène échelonnées. — *C. R. Acad. Sc., Paris.* 233: 1656. 1951.
- La fixation de l'anhydride carbonique par les feuilles de *Bryophyllum Daigremontianum* Berger, à l'obscurité, en présence de tensions d'oxygène variées. — *C. R. Acad. Sc., Paris.* 236: 313. 1953.
- Ochoa, S.: Biosynthesis of dicarboxylic and tricarboxylic acids by carbon dioxide fixation. — *Symp. of Soc. exptl. Biol. V. Cambridge University Press.*: 29. 1951.
- Carbon dioxide fixation in animals and plants. — *Bull. Soc. Chim. biol.* 35: 83. 1953.
- Plantefol, L.: Les eudiomètres de précision. Description d'un appareil universel et technique des analyses. — *Bull. Soc. Chim. biol.* 14: 929. 1932.
- Un eudiomètre simple. — *Bull. Soc. Chim. biol.* 15: 992. 1933.
- Sur les échanges respiratoires des tissus végétaux en culture. — *C. R. Acad. Sc., Paris.* 207: 1121. 1938.
- & Moyse, A.: Un appareil universel d'extraction des gaz et un manomètre comparateur, utilisés dans l'analyse des échanges gazeux. *Bull. Soc. Chim. biol.* 30: 121. 1948.
- Pucher, G. W., Leavenworth, C. S., Ginter, W. D. & Vickery, H. B.: Studies in the metabolism of Crassulacean plants: changes in the composition of *Bryophyllum calycinum* during growth. — *Plant Physiol.* 22: 1. 1947 a.
- , —, —: Studies in the metabolism of Crassulacean plants: the diurnal variation in organic acid and starch content of *Bryophyllum calycinum*. — *Plant Physiol.* 22: 360. 1947 b.
- , —, —: Studies in the metabolism of Crassulacean plants: the behavior of excised leaves of *Bryophyllum calycinum* during culture in water. — *Plant Physiol.* 22: 477. 1947 c.
- , —, —: Studies in the metabolism of Crassulacean plants: the effect of temperature upon the culture of excised leaves of *Bryophyllum calycinum*. — *Plant Physiol.* 23: 123. 1948.
- , Vickery, H. B., Abrahams, M. D. & Leavenworth, C. S.: Studies in the metabolism of

- Crassulacean plants: diurnal variation of organic acids and starch in excised leaves of *Bryophyllum calycinum*. — *Plant Physiol.* 24: 610. 1949.
- Purjevitch, K.: Die Bildung und Zersetzung der organischen Säuren bei den höheren Pflanzen. — *Bot. Zentralb.* 58: 368. 1893.
- Ranson, S. L.: Zymasis and acid metabolism in higher plants. — *Nature.* 172: 252. 1953.
- Richards, H. M.: Acidity and gas exchange in Cacti. — *Carnegie Inst. Washington Publ.* 209. 1915.
- Somers, G. F.: The influence of light, temperature, and some enzyme poisons upon the total organic acid content of leaf tissue of *Kalanchoë Daigremontiana* (Hamet and Perrier). — *Plant Physiol.* 26: 1. 1951.
- Stutz, R. E. & Burris, R. H.: Photosynthesis and metabolism of organic acids in higher plants. — *Plant Physiol.* 26: 226. 1951.
- Taylor, D. L.: Effects of oxygen on respiration, fermentation and growth in wheat and rice. — *Science.* 95: 129. 1942.
- Thomas, M.: Physiological studies on acid metabolism in green plants. I — CO_2 fixation and CO_2 liberation in Crassulacean acid metabolism. — *New Phytol.* 48: 390. 1949.
- Carbon dioxide fixation and acid synthesis in Crassulacean acid metabolism. — *Symposia of Soc. exptl Biol. V. Carbon dioxide fixation and photosynthesis.* Cambridge Univ. Press: 72. 1951.
- & Beevers, H.: Physiological studies on acid metabolism in green plants. II — Evidence of CO_2 fixation in *Bryophyllum* and the study of diurnal variation of acidity in this genus. — *New Phytol.* 48: 421. 1949.
- & Ranson, S. L.: Physiological studies on acid metabolism in green plants. III — Further evidence of CO_2 fixation during dark acidification of plants showing Crassulacean acid metabolism. — *New Phytol.* 53: 1. 1954.
- Thurlow, J. & Bonner, J.: Fixation of atmospheric CO_2 in the dark by leaves of *Bryophyllum*. — *Arch. Biochem.* 19: 509. 1948.
- Truhaut, R.: Un des grands problèmes actuels de biologie générale. L'assimilation de l'anhydride carbonique par les êtres hétérotrophes. La β -carboxylation. — *Biol. méd.* 42: 51. 1953.
- Umbreit, W. W., Burris, R. H. & Stauffer, J. F.: *Manometric techniques and tissue metabolism.* — Burgess Publ. Co ed. Minneapolis USA, 7ème ed. 1951.
- Varner, J. E. & Burrell, R. C.: Use of ^{14}C in the study of the acid metabolism of *Bryophyllum calycinum*. — *Arch. Biochem.* 25: 280. 1950.
- Vennesland, B.: The β -carboxylases of plants. II. The distribution of oxalacetic carboxylase in plant tissues. — *Journ. biol. Chem.* 178: 591. 1949.
- & Conn, E. E.: Carboxylating enzymes in plants. — *Ann. Rev. Plant Physiol.* 3: 307. 1952.
- , Gollub, M. C. & Spek, J. F.: The β -carboxylases of plants. I — Some properties of oxalacetic carboxylase and its quantitative assay. — *Journ. biol. Chem.* 178: 301. 1949.
- Vickery, H. B.: The behavior of isocitric acid in excised leaves of *Bryophyllum calycinum* during culture in alternating light and darkness. — *Plant Physiol.* 27: 9. 1952 a.
- The formation of starch in leaves of *Bryophyllum calycinum* cultured in darkness. — *Plant Physiol.* 27: 231. 1952 b.
- Vlavis, J. & Davis, A. R.: Germination, growth and respiration of rice and barley seedlings at low oxygen pressures. — *Plant Physiol.* 18: 685. 1943.
- Warburg, O.: Ueber die Bedeutung der organischen Säuren für den Lebensprozess der Pflanzen (Speziell der sog. Fettpflanzen). — *Untersuch. Bot. Inst. Tübingen.* 2: 53. 1886.

- Wolf, J.: Beitrag zur Kenntnis der Säurestoffwechsels succulenter Crassulacean. — *Planta*. 15: 572. 1931/1932.
- Beiträge zur Kenntnis des Säurestoffwechsels sukkulenter Crassulaceen II — Untersuchungen über Beziehungen zwischen Sedoheptose und Aepfelsäure. — *Planta*. 26: 516. 1937.
- Beiträge zur Kenntnis des Säurestoffwechsels sukkulenter Crassulaceen. VI. — Neuere Vorstellungen vom Chemismus des Säurestoffwechsels. *Planta*. 37: 510. 1949.
- Wood, H. G., A consideration of some reactions involving carbon dioxide fixation. — *Symp. of Soc. exptl. Biol. V. Carbon dioxide fixation and photosynthesis*. Cambridge Univ. Press. 9. 1951.

Le métabolisme des acides organiques chez le *Bryophyllum* (Crassulacée)

II. Les variations de l'acidité et la photosynthèse, en fonction de la tension d'oxygène

Par

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A. Introduction

Dans un précédent article, j'ai décrit l'influence qu'exerce l'oxygène sur la fixation de l'anhydride carbonique par les feuilles de *Bryophyllum Dairgremontianum* Berger et sur l'acidité de ces feuilles. (Moise, 1955).

L'étude des variations nycthémérales de l'acidité des plantes grasses a montré que la photosynthèse est un des facteurs déterminant leur grandeur. Plusieurs hypothèses ont été émises sur les relations qui peuvent exister entre l'appauvrissement en acide malique et l'émission photosynthétique d'oxygène. Mayer (1878, 1899) a supposé qu'après décarboxylation une molécule d'acide malique libère une molécule de CO_2 repris dans la photosynthèse, le reste tricarboné de la molécule d'acide se condensant avec des restes semblables pour synthétiser directement un glucide.

Wolf (1931, 1932) a admis que le même mécanisme de disparition partielle de l'acide malique intervient à l'obscurité, après 24 h., et à la lumière immédiatement, par décarboxylation, formation d'acide pyruvique et de CO_2 . La tension de ce dernier corps qui est repris dans la photosynthèse reste faible. La décarboxylation l'emporte constamment. Les restes incomplètement oxydés de l'acide malique favoriseraient la décarboxylation aussi bien à la lumière qu'à l'obscurité.

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La resynthèse des glucides, aussi bien à l'obscurité qu'à la lumière, lorsque le taux d'acide malique diminue, a été soulignée par Bennet-Clark (1933 a et b).

Thomas (1949) a expliqué l'enrichissement en acides des feuilles de *Crasulacées* par une réaction de Wood et Werkman. Le CO_2 libéré par les oxydations respiratoires est fixé par β -carboxylation, d'où genèse d'acide malique à l'obscurité.

Cette réaction nécessite la présence de CO_2 en quantité suffisante. Elle a lieu si la tension partielle de CO_2 dans l'atmosphère interne des tissus est assez élevée.

Si cette tension est très basse, la décarboxylation l'emporte. A la lumière, la photosynthèse réduit la tension partielle de CO_2 de telle manière que la carboxylation n'a pas lieu. Il admet que les activités enzymatiques qui assurent la carboxylation et la décarboxylation peuvent s'exercer aussi bien à la lumière qu'à l'obscurité. La compétition entre la photosynthèse et la carboxylation vis-à-vis du CO_2 disponible permet alors de comprendre comment la carboxylation l'emporte au cours de la nuit, la décarboxylation dominant par contre à la lumière.

Thomas et ses élèves en ont fourni la preuve en plaçant des feuilles de *Bryophyllum* dans des atmosphères d'air enrichi en CO_2 . Le mécanisme de la photosynthèse se trouvant saturé par l'abondance du CO_2 , une partie de ce dernier est disponible pour la carboxylation. Dans ces conditions, on assiste au retard de la décarboxylation à la lumière et même à une fixation active de CO_2 par carboxylation avec enrichissement des tissus en acides (1949, 1954).

J'ai étudié l'influence de l'oxygène sur la désacidification des feuilles de *Bryophyllum Daigremontianum* Berger, à la lumière, et sur leurs échanges gazeux photosynthétiques.

Le rôle de l'oxygène a de multiples aspects puisqu'il accélère ou ralentit l'acidification des feuilles à l'obscurité, selon sa tension partielle et la durée des périodes d'examen, et puisqu'il agit directement sur l'intensité de la photosynthèse.

B. Méthodes & techniques

Des feuilles, aussi identiques que possible, sont prélevées le matin, alors qu'elles sont riches en acides organiques. Elles sont réparties en lots homogènes. Les lots sont pesés et placés dans des enceintes dont l'air est remplacé par des mélanges d' O_2 , de CO_2 et de N_2 en proportions déterminées.

Les enceintes sont mises en présence de lumière artificielle (tubes fluores-

cents Philips, type « lumière du jour »). L'intensité lumineuse, au niveau des feuilles, est de 2.000 lux. Les gaz sont ensuite extraits et analysés.

La réalisation des atmosphères, leur contrôle, l'extraction et l'analyse des gaz, ainsi que les dosages de l'acidité libre et des glucides sont faits comme il a été indiqué précédemment (Moïse, 1955).

C. Expériences & résultats

I — La photosynthèse et la désacidification, en fonction de la tension partielle de l'oxygène dans l'atmosphère

Deux séries d'expériences ont été faites, les feuilles étant mises en présence d'atmosphères de compositions diverses :

— azote purifié ($O_2 < 0,1 \%$), mélanges d'oxygène et d'azote, O_2 présentant en volume les proportions suivantes : 1, 2, 5, 10, 21 (correspondant sensiblement à l'air ordinaire), 50 pour cent, et oxygène purifié.

La courbe de la figure I a représenté les résultats moyens obtenus les 24 et 25 Octobre 1952, avec des feuilles n° 3 de $10 \text{ cm} \pm 1 \text{ cm}$ de longueur, cueillies à 8 h. le matin et exposées à la lumière pendant 10 heures, la température étant de 19°C .

L'émission d' O_2 , en 10 h., est de $4,5 \text{ cm}^3$ par g. de substance sèche, dans l'azote. Elle s'élève à $8,03 \text{ cm}^3$ (maximum) dans l'atmosphère $O_2 = 5$ pour cent, conserve une valeur élevée jusqu'à la tension partielle d' O_2 de 50 pour cent ($7,71 \text{ cm}^3$) et diminue brusquement dans l'oxygène pur ($3,26 \text{ cm}^3$).

Il est curieux de constater simultanément une émission de CO_2 , ce qui confirme les anciennes observations de Aubert (1892). Cette émission est très faible dans l'azote : moins de $0,1 \text{ cm}^3$ en 10 heures, par g. de substance sèche, et croît régulièrement lorsque la tension partielle d' O_2 s'élève. Elle atteint $2,89 \text{ cm}^3$ dans l'atmosphère initiale d' O_2 pur, avec une tension partielle de 0,9 pour cent.

Des analyses de contrôle des glucides ont été faites sur des feuilles témoins

Tableau 1. Contrôle glucidique. Glucides exprimés en mg. de glucose par g. de substance sèche.

	a) Réducteur libre	b) Réd. après hydrolyse par l'invertase	c) Réd. après action de SO_4H_2 0,35 N	b)—a) Saccharose	b)—c) Sédo- heptose	d) Amidon	Glucides totaux
Début d'expé- rience	6,9	22,3	19,2	15,4	3,1	22,7	45
Fin d' exp. après 11 h. illumination	10,7	21,4	19,3	10,7	2,1	28,5	49,9

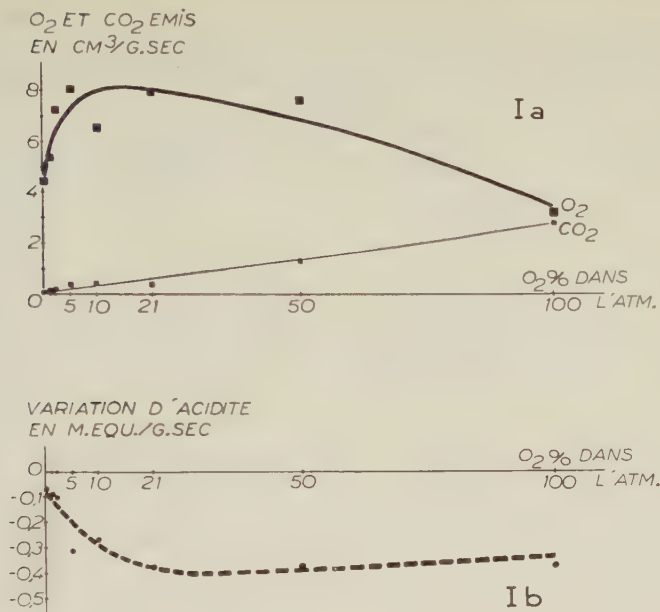


Figure 1. Série 1 : Feuilles de *Bryophyllum Daigremontianum* Berger.

I a. — Variations de l'émission d'O₂ par photosynthèse et de l'émission de CO₂ par oxydation, en fonction de la tension partielle d'oxygène. (O₂ émis et CO₂ émis : cm³ par g. de substance sèche).

I b. — Variations de l'acidité libre (pertes), (acidité exprimée en milli-équivalents par g. de substance sèche) : Acidité initiale=1,30 m. éq./g. sec.

Pression partielle initiale de CO₂=0. Durée de l'expérience : 10 h. Eclairement : 2.000 lux. T=19° C.

cueillies en même temps que les précédentes et fixées soit immédiatement, soit après 11 h. d'illumination à 2.000 lux, à l'air libre. Le tableau I indique les résultats de ces analyses.

L'accroissement du taux glucidique est faible. Il est dû au glucose (Réducteur libre — sédoheptose) et à l'amidon. Le taux de saccharose et celui du sédoheptose diminuent légèrement. (cf. Pucher et coll., 1947.)

La courbe de la figure 1 b présente les pertes d'acidité des feuilles en fonction de la tension d'O₂. L'acidité initiale des feuilles est de 1,30 m. éq. Les diminutions de l'acidité à la lumière sont dues vraisemblablement à celles du taux d'acide malique principalement, et, à un moindre degré, à celles du taux d'acide citrique, ainsi que l'ont établi Pucher et coll. (1947, 1949) pour les feuilles de *Bryophyllum calycinum*. L'acide isocitrique, par contre, ne présente pas de variations sensibles lors des alternances de jours et de nuits (Vickery, 1952).

Tableau 2. Feuilles n°3 de *Bryophyllum Daigremontianum* Berger, prélevées le 28 Octobre 1952, à 7 h. du matin. — $\text{CO}_2=0$. $T=19^\circ \text{C}$. Eclairement : 2.000 lux. Durée : 9 h. \pm 0 h. 30. O_2 et CO_2 émis en cm^3 par g. de substance sèche, perte d'acidité en milli-équivalents par g. de substance sèche.

Atmosphères initiales	O_2 émis		CO_2 émis	Pertes d'acidité	
N	$\left\{ \begin{array}{l} 1 \\ 2 \\ 3 \end{array} \right.$	$\left\{ \begin{array}{l} 4,8 \\ 4,0 \\ 6,3 \end{array} \right.$ Moyenne 5,0	$\left\{ \begin{array}{l} 0 \\ 0 \\ 0 \end{array} \right.$	$\left\{ \begin{array}{l} 0,45 \\ 0,42 \\ 0,48 \end{array} \right.$	Moyenne 0,45
$\text{O}_2 = 21 \%$	$\left\{ \begin{array}{l} 1 \\ 2 \\ 3 \end{array} \right.$	$\left\{ \begin{array}{l} 6,2 \\ 6,4 \\ 7,1 \end{array} \right.$ Moyenne 6,6	$\left\{ \begin{array}{l} 0,4 \\ 0,4 \\ 0,3 \end{array} \right.$ Moyenne 0,4	$\left\{ \begin{array}{l} 0,51 \\ 0,51 \\ 0,48 \end{array} \right.$	Moyenne 0,50
$\text{O}_2 = 100 \%$	$\left\{ \begin{array}{l} 1 \\ 2 \\ 3 \end{array} \right.$	$\left\{ \begin{array}{l} 1,8 \\ 1,8 \\ 3,0 \end{array} \right.$ Moyenne 2,2	$\left\{ \begin{array}{l} 1,3 \\ 1,1 \\ 1,3 \end{array} \right.$ Moyenne 1,2	$\left\{ \begin{array}{l} 0,30 \\ 0,37 \\ 0,47 \end{array} \right.$	Moyenne 0,38
Témoins à l'air libre					0,55

Les pertes sont faibles dans l'azote : 0,07 m. éq. par g. de substance sèche en 10 h., mais croissent très rapidement lorsque la tension d' O_2 s'élève. Elles sont de 0,31 dans l'atmosphère d'oxygène à 5 pour cent et continuent à croître, bien que plus lentement, en présence des atmosphères plus riches en O_2 . Elles atteignent 0,38 dans l'atmosphère $\text{O}_2=21$ pour cent et ne s'élèvent plus ensuite.

Des feuilles témoins, éclairées dans les mêmes conditions que les précédentes, mais à l'air libre, présentent une perte d'acidité beaucoup plus forte, s'élevant à 0,68 m. éq. par g. de substance sèche. On peut penser que la propre tension de CO_2 réalisée dans les atmosphères confinées au cours des expériences limite la perte d'acidité par une reprise de la carboxylation.

Une expérience faite en utilisant seulement trois atmosphères, l'une d'azote, l'autre d'azote et d'oxygène à 21 pour cent, la troisième d'oxygène pur, confirme cette interprétation ainsi que les autres résultats des expériences précédentes (Tableau 2). On y retrouve les mêmes variations dans les émissions d' O_2 pur et de CO_2 et, de plus, une perte d'acidité maximum, non plus en présence d' O_2 , mais dans l'air ordinaire. Dans l'oxygène pur, le dégagement abondant de CO_2 provoque vraisemblablement une reprise active de la carboxylation.

Dans toutes ces expériences la teneur en eau des feuilles s'échelonne de 1150 à 1250 pour 100 de substance sèche et les variations des échantillons, selon les atmosphères dans lesquelles ils ont séjourné, ne sont pas significatives. Les variations du poids de substance sèche sont trop faibles pour être décelées et peuvent être négligées dans l'interprétation des résultats.

Les conclusions suivantes se dégagent de ces résultats

1. — En l'absence de CO_2 externe, la photosynthèse a lieu aux dépens du carbone des acides organiques dont la quantité diminue.

Tableau 3. Valeurs du quotient photosynthétique apparent ($Q. P. = \frac{O_2 \text{ émis}}{CO_2 \text{ absorbé}}$) en fonction de la tension partielle d' O_2 , la tension de CO_2 initiale étant nulle. (Exp. des 24 et 25 Octobre) — Fig. I a).

Atmos- phères initiales	N ($O_2 < 0,1 \%$)	$O_2 = 1 \%$	$O_2 = 2 \%$	$O_2 = 5 \%$	$O_2 = 10 \%$	$O_2 = 21 \%$	$O_2 = 50 \%$	$O_2 = 100 \%$
Q.P. . .	— 56	— 53	— 52	— 25	— 17	— 23	— 6	— 1,1

La photosynthèse croît en intensité avec l'oxygénation, atteint son maximum quand O_2 est présent à 5 pour cent et se maintient encore à des valeurs élevées jusque dans l' O_2 à 50 pour cent. Elle décroît brusquement dans O_2 pur.

2. — Une émission simultanée de CO_3 se manifeste. En grandeur, elle est fonction de la tension partielle d' O_2 . Dans l'azote, ainsi qu'en présence des très faibles tensions d' O_2 , elle est masquée par la photosynthèse qui reprend le CO_2 à mesure de sa libération. Mais elle devient nettement perceptible quand la tension d' O_2 s'accroît et atteint son maximum dans l' O_2 pur. Elle permet même, dans ces conditions, une nette reprise de la carboxylation qui se traduit par une diminution de la perte d'acidité.

3. — On peut admettre que dans l'azote et dans les atmosphères très faiblement oxygénées, la vitesse même de la photosynthèse est déterminée, en partie au moins, par la vitesse de la décarboxylation ou de l'oxydation des acides organiques. On sait qu'aux basses tensions d' O_2 , la photosynthèse croît lorsque la tension d' O_2 s'élève (Franck, 1935). La photosynthèse atteint ses plus fortes valeurs et les conserve dans la mesure où elle n'est pas elle-même partiellement inhibée par l'oxygène, ainsi que l'ont établi des recherches antérieures (Franck, 1951).

Ces résultats sont également à rapprocher de ceux obtenus par Molliard (1935, 1936) avec le Radis. L'optimum de croissance des plantes et vraisemblablement l'optimum d'activité photosynthétique sont réalisés en présence d'atmosphères renfermant 5 pour cent d' O_2 . Les tensions plus élevées provoquent une chute de croissance d'autant plus prononcée qu'elles sont plus fortes.

4. — L'apparition de CO_2 dans ces expériences montre que le point de compensation lumineux pour la photosynthèse et la décarboxylation est nettement plus élevé que le point de compensation correspondant à la photosynthèse et aux oxydations respiratoires habituelles. Ce dernier est toujours inférieur à 1.000 lux (Rabinowitch, 1951).

5. — Le quotient photosynthétique apparent est négatif, en raison de cette émission de CO_2 , et il est d'autant plus élevé en valeur absolue que la tension d' O_2 est plus basse (Tableau III).

6. — Considérant que les variations d'acidité sont dûes presque exclusivement à celles du taux d'acide malique, il est possible de calculer la perte théorique d'acide en fonction de l'émission d'O₂.

D'après la réaction globale de la réduction de l'acide malique en glucose :



on peut calculer que l'émission de 3 O₂ correspond à une perte d'acidité de 6 milli-équivalents, soit une émission de 11,2 cm³ d'O₂ par milli-équivalent perdu.

Ce calcul, appliqué aux résultats précédents, montre que la perte d'acidité n'atteint un ordre de grandeur compatible avec l'émission d'O₂ qu'à partir de la tension partielle de 5 pour cent.

En présence des tensions d'O₂ inférieures, plus d'O₂ est émis que ne le laissent prévoir les diminutions d'acidité, ce qui conduit à penser que des phénomènes fermentaires interviennent libérant du CO₂ et alimentant ainsi indirectement l'émission d'O₂.

Dans l'O₂ pur, de tout le CO₂ émis par la simple décarboxylation de l'acide malique, un peu plus de la moitié seulement serait repris dans la réduction photosynthétique.

De tels calculs, très approximatifs, ainsi que je l'ai indiqué dans la première partie de ce mémoire, ne permettent pas de conclure avec plus de précision.

7. — A propos du mécanisme même de la photosynthèse, ces résultats ne permettent pas d'établir que *tout* le carbone de l'acide malique doit d'abord être oxydé, passer à l'état de CO₂, avant d'être réduit dans la photosynthèse. Quelles que soient les réactions intermédiaires, les bilans d'échanges de gaz sont en effet les mêmes. Mais ils permettent de penser qu'une partie au moins des groupes carboxyles de l'acide malique sont libérés à l'état de CO₂ avant qu'il présente chez les *Scenedesmus* et la plupart des organismes examinés malique, leur reprise directe reste du domaine de l'hypothèse. Le comportement de l'acide malique, dans ces feuilles, est différent d'ailleurs de celui qu'il présente chez les *Scenedesmus* et la plupart des organismes examinés au cours des recherches sur la photosynthèse, puisqu'habituellement son taux s'élève à la lumière et diminue à l'obscurité (Calvin et coll., 1952). Ses relations avec le mécanisme de la photosynthèse ne sont donc certainement pas comparables.

Les travaux de Fan et coll. (1943), avec *Chlorella pyrenoidosa* cultivée en présence de différents ions organiques (acétate, pyruvate, lactate, succinate, fumarate, malate, citrate et isocitrate) n'ont pas apporté de conclusions positives sur l'utilisation directe des ions organiques dans la photosynthèse.

Plus récemment, Krotkov et coll. (1954) ont montré qu'il était possible que des feuilles de *Nicotiana Tabacum* nourries d'acide acétique marqué CH₃-¹⁴COOH assimilent le carbone de cet acide partiellement après son oxydation

Tableau 4. Contrôle glucidique. Glucides exprimés en mg. de glucose par g. de substance sèche.

	a) Réducteur libre	b) Réd. après hydrolyse par l'invertase	c) Réd. après action de SO ₄ H ₂ 0,35 N	b)—a) Saccharose	b)—c) Sédo- heptose	d) Amidon	Glucides totaux
Début d'expé- rience	11,0	20,5	13,1	9,5	7,4	18,9	39,4
Fin d'expé- rience après 10 h. d'illu- mination en présence d'air + CO ₂	15,7	21,4	17,8	5,7	3,6	26	47,4

totale et partiellement avant dégradation complète, en raison de la présence de ¹⁴C dans le glucose synthétisé, en positions 2 et 5, alors que la photosynthèse à partir de CO₂ entraîne le marquage rapide en positions 3 et 4 seulement.

Aucun argument probant ne semble encore être apporté sur l'utilisation directe de chaînons tricarbonés dans la photosynthèse.

II. La photosynthèse et l'équilibre de l'acidité, en fonction de la tension partielle de l'oxygène dans l'atmosphère

Deux séries d'expériences, parallèles aux précédentes, ont été faites, en présence des mêmes tensions d'O₂ et, en plus, de CO₂ à la tension partielle de 5,7 ± 0,1 pour cent.

La courbe de la figure 2 a représente les résultats moyens obtenus les 10 et 11 Octobre 1952, avec des feuilles identiques à celles utilisées précédemment, prélevées à 7 h. le matin et soumises aux mêmes conditions de température et d'éclairement.

L'émission d'O₂, en 10 h., est de 8 cm³ par g. de substance sèche, en atmosphère d'azote. Elle croît rapidement lorsque la tension partielle d'oxygène s'élève (13 cm³, 7 pour O₂=1 pour cent), reste stationnaire, puis diminue dès que cette tension atteint 10 pour cent, lentement d'abord (11,1 cm³ pour O₂=21 pour cent), enfin rapidement (4,9 cm³ pour O₂=94,3 pour cent).

L'absorption de CO₂ présente des variations de même allure. De 8,1 cm³ dans l'azote, elle s'élève (12,8 cm³ pour O₂=1 pour cent), puis reste stationnaire jusqu'à ce que la tension d'O₂ dépasse 21 pour cent. Elle diminue alors, mais moins rapidement que l'émission d'O₂. La tension finale de CO₂ dans les atmosphères ne s'est jamais abaissée au-dessous de 1,8 pour cent.

Le contrôle des glucides a donné les résultats suivants (Tableau 4). Il a été effectué avec des feuilles qui ont séjourné pendant la même durée que

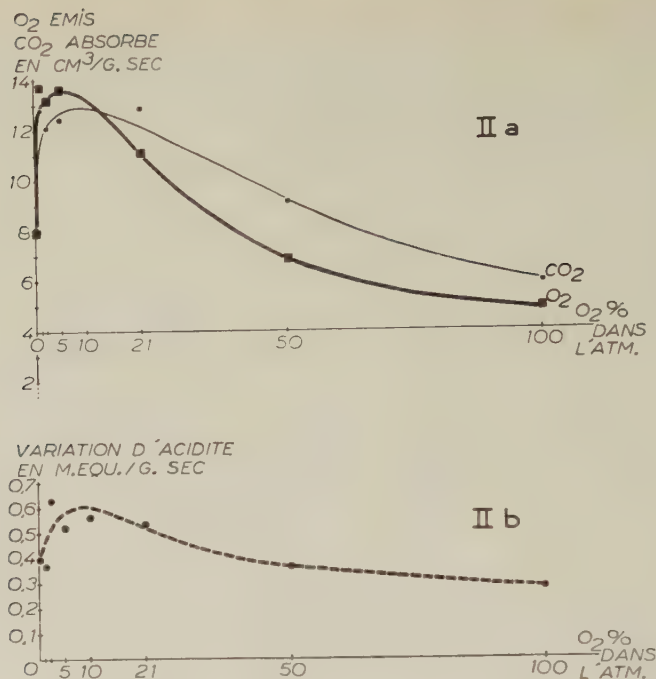


Figure 2. — Série 2 : Feuilles de *Bryophyllum Daigremontianum* Berger.

II a. — Variations de l'émission d'O₂ par photosynthèse et de l'absorption de CO₂ en fonction de la tension partielle d'oxygène. (O₂ émis et CO₂ absorbé : cm³ par g. de substance sèche.)

II b. — Variation de l'acidité libre (gains), (acidité exprimée en milli-équivalents par g. de substance sèche).

Pression partielle initiale de CO₂ = 5,7 %. — Durée de l'expérience : 10 h. Eclairement : 2.000 lux. T = 19° C.

les précédentes dans une atmosphère d'air enrichi de 6 pour cent de CO₂ et ont été illuminées dans les mêmes conditions.

L'accroissement du taux glucidique, plus élevé que dans l'expérience précédente, est dû à celui du glucose (réducteur libre — sédoheptose) et à celui de l'amidon. Les diminutions des taux de saccharose et de sédoheptose sont également sensibles.

La courbe de la figure 2 b présente les variations correspondantes de l'acidité. L'acidité des feuilles témoins, au début de l'expérience, est de 1,53 m. équ. Il n'y a pas pertes, mais gains d'acidité, ce qui confirme les résultats de Thomas et coll. (1949, 1954).

Ces gains, de 0,41 m. équ. dans l'azote, augmentent d'abord lorsque la tension d'O₂ s'élève (0,63 m. équ. en présence d'O₂ = 2 pour cent) puis se stabilisent et ne diminuent que lorsque la tension d'O₂ dépasse 21 pour cent.

Tableau 5. Feuilles n°3 de *Bryophyllum Daigremontianum* Berger, prélevées le 4 Novembre 1952 à 7 h. du matin. $\text{CO}_2=6$ pour cent. T 19° C. Eclairement : 2.000 Lux. Durée : 10 h. ± 0 h 30. O_2 émis et CO_2 absorbé en cm^3 par g. de substance sèche, gains d'acidité en milli-équivalents par g. de substance sèche

Atmosphères initiales	O_2 émis		CO_2 absorbé	Quotient photo-synthétique apparent	Variations de l'acidité
$\text{N}_2 = 94,1 \%$ ($\text{O}_2 < 0,1 \%$) + $\text{CO}_2 = 5,9 \%$	$\left\{ \begin{array}{l} 1 \\ 2 \\ 3 \end{array} \right.$	$\left\{ \begin{array}{l} 10,1 \\ 9,3 \\ 8,6 \end{array} \right.$	Moyenne 9,3	$\left\{ \begin{array}{l} 10,0 \\ 9,4 \\ 8,8 \end{array} \right.$ Moyenne 9,4	$\left\{ \begin{array}{l} -0,03 \\ -0,03 \\ -0,06 \end{array} \right.$ Moyenne $-0,04$
$\text{O}_2 = 20 \%$ + $\text{CO}_2 = 6 \%$	$\left\{ \begin{array}{l} 1 \\ 2 \\ 3 \end{array} \right.$	$\left\{ \begin{array}{l} 10,5 \\ 12,1 \\ 11,6 \end{array} \right.$	Moyenne 11,4	$\left\{ \begin{array}{l} 11,0 \\ 12,3 \\ 11,5 \end{array} \right.$ Moyenne 11,6	$\left\{ \begin{array}{l} -0,11 \\ -0,09 \\ -0,08 \end{array} \right.$ Moyenne $-0,09$
$\text{O}_2 = 94,1 \%$ + $\text{CO}_2 = 5,9 \%$	$\left\{ \begin{array}{l} 1 \\ 2 \\ 3 \end{array} \right.$	$\left\{ \begin{array}{l} 7,4 \\ 6,0 \\ 7,1 \end{array} \right.$	Moyenne 6,8	$\left\{ \begin{array}{l} 8,3 \\ 6,6 \\ 7,6 \end{array} \right.$ Moyenne 7,5	$\left\{ \begin{array}{l} -0,26 \\ -0,35 \\ -0,14 \end{array} \right.$ Moyenne $-0,25$

Le gain n'est plus que de 0.28 m. éq. dans l'atmosphère la plus riche en O_2 . Les feuilles témoins, laissées à l'air libre, ont perdu pendant le même temps 0,41 m. éq.

Une autre expérience a été faite avec des feuilles comparables mises en présence d'atmosphères renfermant 6 pour cent de CO_2 et soit de l'azote, soit un mélange d'azote et d'oxygène ($\text{O}_2=20$ pour cent), soit de l'oxygène pur. Les résultats en sont exprimés dans le tableau 5.

Dans toutes ces expériences, la teneur en eau des feuilles s'échelonne de 1230 à 1370 pour 100 de substance sèche.

Les feuilles témoins, laissées à l'air libre, ont perdu pendant le même temps 0.42 m. éq. Si, dans l'expérience présente, il n'y a pas gain, mais perte d'acidité, cette perte est nettement moins élevée que celle subie par les témoins.

Les conclusions suivantes peuvent être dégagées :

1. — Lorsque le CO_2 se trouve en abondance dans l'atmosphère, la photosynthèse a lieu à ses dépens. De plus, grâce à lui, la désacidification est ralentie, annulée ou bien même remplacée par une acidification nette, ainsi que l'ont montré Thomas et coll. (1949, 1954).

Si l'on compare les deux séries d'expériences, la première faite avec des atmosphères initialement dépourvues de CO_2 , la seconde avec des atmosphères riches en CO_2 , on constate que, dans le second cas, la photosynthèse est beaucoup plus intense que dans le premier. Elle est environ 2 fois plus rapide.

L'excédent de CO_2 disponible peut même accroître la vitesse de la carboxylation, ce que traduit l'élévation de l'acidité des feuilles.

2. — La photosynthèse et la fixation de CO_2 par β -carboxylation croissent

Tableau 6. Valeurs du quotient photosynthétique apparent ($Q. P. = \frac{O_2 \text{ émis}}{CO_2 \text{ absorbé}}$) en fonction de la tension partielle d'O₂ et en présence de CO₂ abondant ($5,7 \pm 0,1$ pour cent). (Exp. des 10 et 11 Octobre 1952 — Fig. II a).

Atmos- phères initiales	N (O ₂ < 0,1 %)	O ₂ = 1 %	O ₂ = 2 %	O ₂ = 5 %	O ₂ = 10 %	O ₂ = 21 %	O ₂ = 50 %	O ₂ = 94,3 %
Q.P. ...	0,98	1,07	1,09	1,09	1,05	0,87	0,75	0,83

avec l'oxygénation aux basses tensions d'O₂. Dans l'azote, la photosynthèse est peu intense ainsi qu'il est généralement reconnu chez les plantes. Il en est de même de la carboxylation.

L'intensité de la photosynthèse et celle de la carboxylation s'élèvent quand les atmosphères renferment initialement de 1 à 5 pour cent d'O₂. En présence de tensions d'O₂ plus élevées, la photosynthèse se ralentit. Il en est de même de la carboxylation et l'on constate simultanément l'inhibition de la photosynthèse et la tendance à la désacidification lorsque s'élève la tension d'O₂.

Ces résultats confirment sur ces divers points les résultats obtenus précédemment.

3. — Le quotient photosynthétique apparent est positif et très voisin de l'unité (Tableaux 5 et 6). Il est d'autant plus proche de cette dernière valeur que le taux d'acidité est plus stable, ainsi qu'on le constate notamment en présence des faibles tensions d'O₂, alors que les oxydations, la carboxylation et la décarboxylation sont lentes. (cf. Ranson, in Thomas, 1951, Moyse, 1953).

Aux tensions élevées d'O₂, la photosynthèse est plus déprimée que ne l'est la carboxylation, l'émission photosynthétique d'O₂ est plus amoindrie que ne l'est la fixation de CO₂, et le Q. P. apparent est alors légèrement inférieur à l'unité.

D'après la réaction globale de fixation de CO₂ par carboxylation ou la réaction inverse de décarboxylation :



on peut calculer la part qui revient à cette réaction dans la fixation totale de CO₂ à la lumière.

Dans le cas où la variation d'acidité est la moins forte, (Tableau V), on constate que le Q. P. corrigé reste encore très voisin de 1 (0,94 pour l'atmosphère N₂=94,1 pour cent, 0,91 pour l'atmosphère O₂=20 pour cent), mais il s'en éloigne quand la variation d'acidité est plus intense (0,66 pour l'atmosphère O₂=94,1 pour cent).

La part des phénomènes simultanés : photosynthèse, oxydations des glucides, carboxylation et décarboxylation, ne peut être établie avec précision dans l'ignorance d'un certain nombre de facteurs tels que la grandeur réelle des oxydations glucidiques, la répartition du CO_2 fixé par la photosynthèse et la carboxylation.

D. Conclusion

La compétition pour l'anhydride carbonique entre la photosynthèse et la carboxylation se trouve bien établie pour les feuilles de Crassulacées.

La valeur de la tension partielle de CO_2 réalisée par les oxydations cellulaires, suffisante pour que la carboxylation domine à l'obscurité, ne l'est plus à la lumière, quand la photosynthèse intervient avec son mécanisme rapide d'assimilation carbonée. La soustraction rapide du CO_2 ne permet plus à la réaction de carboxylation d'équilibrer la décarboxylation qui l'emporte alors. Un apport supplémentaire de CO_2 peut, assurant la saturation du mécanisme photosynthétique, renverser le sens de l'équilibre qui gouverne l'acidité des feuilles.

Dans la physiologie naturelle des Crassulacées, la β -carboxylation réalise une conservation immédiate du CO_2 d'origine respiratoire et une fixation nocturne du CO_2 atmosphérique. La décarboxylation réalise pendant le jour la tension de CO_2 tissulaire nécessaire pour une photosynthèse intense.

Les deux mécanismes de fixation et de réduction de CO_2 se relaient dans le temps grâce à l'activité β -carboxylasique beaucoup plus intense que celle qui est reconnue chez d'autres plantes.

A ce propos, Koursanov (1954) a montré que chez le Haricot, la carboxylation peut, au niveau des racines, jouer un rôle non négligeable dans la fixation totale de CO_2 par la plante entière.

La physiologie des plantes grasses diffère en ce que les feuilles présentent une activité carboxylasique suffisamment intense pour masquer toute émission du CO_2 respiratoire, assurer une fixation nocturne du CO_2 externe, de telle sorte que la photosynthèse peut, à la lumière, présenter ensuite une intensité beaucoup plus élevée que celle qu'elle atteindrait par une simple compensation avec la respiration en l'absence de CO_2 externe.

Le rôle de l'oxygène dans les oxydations glucidiques, l'acidification et la désacidification, et la photosynthèse, est primordial.

Pour les feuilles de Bryophyllum, la multiplicité de ses actions peut s'analyser ainsi :

a) *Oxydations cellulaires.* L'oxygène les accélère vivement. Aux confins de l'anaérobiose, il assurerait la désintoxication des cellules vis-à-vis des produits fermentaires, inhibiteurs de la carboxylation et de la photosynthèse.

b) *Acidification*. L'oxygène accélère la fixation de l'anhydride carbonique par la genèse d'un substrat tricarboné qui peut être carboxylé et conduire ainsi à l'accumulation d'acide malique. Cette action est sensible à l'obscurité et à la lumière.

c) *Désacidification*. L'oxygène accélère à l'obscurité, comme à la lumière, l'oxydation des acides organiques, avec émission d'anhydride carbonique, la désacidification commençant très vraisemblablement par une décarboxylation.

Selon que les feuilles sont riches ou pauvres en acides, l'action de l'oxygène favorise immédiatement soit la carboxylation, soit la décarboxylation. Pouvant agir sur les deux mécanismes opposés de la même manière, en accroissant leur vitesse, il ne peut être le seul facteur qui règle le sens du déplacement de l'équilibre entre ces deux mécanismes. Son action, même conjuguée avec celle de l'anhydride carbonique, ne peut expliquer d'une manière satisfaisante le renversement de l'activité enzymatique à l'obscurité, lorsque les feuilles ont atteint leur maximum d'acidité. L'étude de la cinétique de la carboxylation et de la décarboxylation, en présence des produits qui résultent de ces réactions inverses, pourraient peut-être apporter des renseignements sur cette question.

d) *Photosynthèse*. L'oxygène agit sur la vitesse de la photosynthèse, en accélérant les oxydations génératrices d'anhydride carbonique lorsque les feuilles ne peuvent en trouver dans l'atmosphère externe.

Parmi les diverses actions que l'oxygène peut exercer sur la photosynthèse aux confins de l'anaérobiose, il faut donc tenir compte du rôle qu'il joue dans l'apport d'anhydride carbonique en favorisant l'oxydation des acides organiques.

Mais son action sur la photosynthèse se traduit aussi par une inhibition, dès que sa tension partielle atteint des valeurs élevées dans l'atmosphère.

Les connections qui existent entre les oxydations cellulaires, les mécanismes de l'acidification et de la désacidification des feuilles et enfin la photosynthèse sont établies à la fois par l'intervention des mêmes produits minéraux tels que l'anhydride carbonique, l'oxygène et l'eau et par un certain nombre d'acides organiques communs qui appartiennent au métabolisme intermédiaire. La concentration de chacune de ces substances gouverne le sens de ces réactions diverses dans leurs résultats, selon la prédominance des activités enzymatiques nécessaires aux synthèses comme aux dégradations. Des travaux sont en cours pour préciser ces points, par l'analyse des principaux acides organiques présents dans les feuilles de *Bryophyllum*.

Résumé

Les échanges de gaz photosynthétiques et les variations de l'acidité des feuilles de *Bryophyllum Daigremontianum* Berger (Crassulacée) sont étudiés

soit en présence, soit en l'absence de CO_2 externe, dans des atmosphères dont les tensions d'oxygène s'échelonnent de 0 à 100 pour cent.

En l'absence de CO_2 externe, la dégradation des acides organiques assure la fourniture d'aliment carboné à la photosynthèse.

Les faibles tensions d' O_2 qui favorisent une vitesse très appréciable de la désacidification et une vitesse élevée du mécanisme photosynthétique permettent à la photosynthèse d'atteindre son intensité maximum.

Les fortes tensions d' O_2 , quoique très favorables aux oxydations des glucides et des acides organiques, inhibent partiellement la photosynthèse.

En présence de CO_2 externe abondant, l'acidification peut se poursuivre à la lumière concurremment à la photosynthèse. L'action favorable des faibles tensions d' O_2 se manifeste également. Les hautes tensions d' O_2 dépriment plus la photosynthèse que la fixation de CO_2 par β -carboxylation et l'acidification qui en résulte.

Les différents mécanismes physiologiques mis en jeu : oxydations glucidiques, carboxylation et décarboxylation et enfin photosynthèse sont confrontés en présence ou en l'absence de CO_2 , en fonction de la tension partielle d'oxygène.

Je remercie vivement Mr. le Professeur M. Thomas, du King's College (Newcastle upon Tyne), qui m'a prodigué du précieux conseils et m'a fait profiter de ses suggestions critiques.

Bibliographie

- Aubert, E.: Recherches sur la respiration et l'assimilation des plantes grasses. Chap. II. — Rev. gén. Bot. 4: 386. 1892.
- Bennet-Clark, T. A.: The rôle of the organic acids in plant metabolism. — New Phytol. 32: 37. 1933 a, 32: 128. 1933 b.
- Calvin, M. & Massini, P.: The path of carbon in photosynthesis — XX The steady state Experimentia 8: 445. 1952.
- Fan, C. S., Stauffer, J. F. & Umbreit, W. W.: An experimental separation of oxygen liberation from carbon dioxide fixation in photosynthesis by *Chlorella*. — Journ. gen. Physiol. 27: 15. 1943.
- Franck, J.: Notizen zur Photosynthese: Chem. Rev. 17: 433. 1935.
- A critical survey of the physical background of photosynthesis. — Ann. Rev. Plant Physiol. 2: 53. 1951.
- Koursanov, A. L.: Circulation des matières organiques dans la plante et action du système racinaire. — Essais de Botanique, Acad. Sc. U.R.S.S. éd.: 129. 1954.
- Kroftkov, G., Vittorio, P. V. & Reed, G. B.: Synthesis of glucose and starch by Tobacco leaves from HC^{14}OOH , $\text{CH}_3\text{C}^{14}\text{OOH}$, $\text{CH}_3\text{—CHOH—C}^{14}\text{OOH}$, $\text{C}^{14}\text{H}_3\text{—C}^{14}\text{HOH—COOH}$ and $\text{C}_6\text{H}_5\text{C}^{14}\text{OOH}$. — Arch. Biochem. Biophys. 51: 147. 1954.
- Mayer, A.: Ueber die Saurestoffausscheidung einiger Crassulaceen. — Landwirt. Versuchst. 21: 277. 1878.
- Neue Untersuchungen über die Crassulaceenapfelsäure und deren physiologische Bedeutung. — Landwirt. Versuchst. 51: 336. 1899.

- Molliard, M.: Action d'un enrichissement de l'atmosphère en oxygène sur le développement des plantes. — C. R. Acad. Sc. Paris, 201: 1160. 1935.
- Le rendement des plantes vertes en fonction de la teneur de l'atmosphère en oxygène. — C. R. Acad. Sc. Paris, 203: 8. 1936.
- Moyse, A.: La photosynthèse et le métabolisme des acides organiques chez *Bryophyllum Daigremontianum* Berger. I. — Les échanges gazeux photosynthétiques en présence d'atmosphères aux tensions d'oxygène échelonnées. — C. R. Acad. Sc. Paris, 236: 111. 1953.
- Le métabolisme des acides organiques chez le *Bryophyllum* (Crassulacée). I. — *Physiol. Plant.* 8: 453. 1955.
- Pucher, G. W., Leavenworth, C. S., Ginter, W. D. & Vickery, H. B.: Studies in the metabolism of Crassulacean plants: the diurnal variation in organic acid and starch content of *Bryophyllum calycinum*. — *Plant Physiol.* 22: 360. 1947.
- Vickery, H. B., Abrahams, M. D. & Leavenworth, C. S.: Studies in the metabolism of Crassulacean plants: diurnal variation of organic acids and starch in excised leaves of *Bryophyllum calycinum*. — *Plant Physiol.* 24: 610. 1949.
- Rabinowitch, E. I.: Photosynthesis and related processes II, Part I. — Interscience Publ. N. Y. 1951.
- Thomas, M.: Physiological studies on acid metabolism in green plants. I. CO₂ fixation and CO₂ liberation in Crassulacean acid metabolism. — *New Phytol.* 48: 390. 1949.
- Carbon dioxide fixation and acid synthesis in Crassulacean acid metabolism. — *Symposia of Soc. exptl. Biol.* V. Carbon dioxide fixation and photosynthesis. Cambridge Univ. Press: 72. 1951.
- & Beevers, H.: Physiological studies on acid metabolism in green plants. II — Evidence of CO₂ fixation in *Bryophyllum* and the study of diurnal variation of acidity in this genus. — *New Phytol.* 48: 421. 1949.
- & Ranson, S. L.: Physiological studies on acid metabolism in green plants. III. — Further evidence of CO₂ fixation during dark acidification of plants showing Crassulacean acid metabolism. — *New Phytol.* 53: 1. 1954.
- Vickery, H. B.: The behavior of isocitric acid in excised leaves of *Bryophyllum calycinum* during culture in alternating light and darkness. — *Plant Physiol.* 27: 9. 1952.
- Wolf, J.: Beitrag zur Kenntnis der Säurestoffwechsels succulenter Crassulaceen. — *Planta.* 15: 572. 1931/1932.

Antagonists to the Caffeine-Inhibition of Fungal Growth

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Introduction

It was found some years ago by one of us (Kihlman), that half the growth inhibiting effect of caffeine on the ascomycete *Neurospora crassa* could be reversed by about equimolar concentrations of adenine. Hypoxanthine, and to a lesser degree adenosine, had a similar effect.

Previously, an antagonism between caffeine and adenine had been described by Macht and Schroeder (1930), who observed that the growth of seedlings of *Lupinus albus* was less inhibited by a mixture of adenine and caffeine than by the compounds taken separately. An antagonistic effect of adenine was also observed by these authors in connection with other biological activities of caffeine.

In 1949 Kidder and Dewey published a study of the effect of substituted purines on the growth of the animal microorganism *Tetrahymena geleii*. They found that the dimethylxanthines and trimethylxanthine (caffeine) inhibited growth but that the inhibition could be partly reversed by guanine or other purines. Kidder and Dewey concluded that the methylxanthines interfere with at least two enzyme systems, one of which is a purine system. The latter is believed to be inhibited, because the structural relationship existing between the methylxanthines and the cellular purines (adenine, guanine), makes them compete for active centers at the enzyme surface (antimetabolite hypothesis).

Since the results obtained by Kihlman in *Neurospora* were almost identical with those obtained by Kidder and Dewey in *Tetrahymena*, it seemed reason-

able to assume that the physiological mechanism behind the caffeine-inhibition is also identical in the two cases. However, when later on the antagonism problem in connection with the growth inhibiting effect of caffeine was reinvestigated with the ascomycete *Ophiostoma multiannulatum* as experimental material, facts soon emerged, which were hardly compatible with an explanation in line with the antimetabolite hypothesis. The results of these experiments are described below.

Methods

Ophiostoma was grown in shaken tubes with liquid nutrient solution (»modified medium 3», Fries 1949), where the fungus was propagating by conidial budding. Each tube contained 10 ml. solution which were sterilised by autoclaving. The tubes were then inoculated with thoroughly washed conidia (from a shake-tube culture) suspended in distilled water, 1 ml. suspension being added to each tube. A wild type strain (No. 5a^V) was used as experimental material. After inoculation the tubes were placed on a shaker at +25° C. The growth was daily determined photometrically.

In the experiments with *Neurospora crassa* (a wild type strain) the fungus was cultivated in »Ryan-tubes» (Ryan et al. 1943) with Medium 3 solidified with 1.5 per cent agar. Each tube contained 15 ml. of the medium. The position of the hyphal frontier was noted every day, and an average value of the hyphal growth rate thus obtained.

Experiments and Discussion

The caffeine-inhibition of growth of different strains of *Ophiostoma* has previously been studied by Fries (1950). His experiments showed that growth as a rule was completely inhibited by a caffeine concentration of 0.175 per cent (=c. 8×10^{-3} M). This observation was confirmed by the present experiments. It was further found that the caffeine-inhibition was dependent on the concentration of conidia in the inoculum. The more conidia the inoculum contained, the weaker the inhibition. Table 1 shows how the inhibition is affected when the inoculum is diluted to one a tenth of the original concentration of conidia.

If the nutrient solution in addition to caffeine contains a sufficient amount of adenine, the caffeine-inhibition is partly released. It appears from table 2 that the inhibition of conidial growth produced by 10^{-2} M of caffeine is hardly at all affected until the adenine concentration is equimolar to that of caffeine. After 7 days of incubation, the growth in the presence of 3×10^{-2} M of adenine proved to be 73 per cent of the growth in the control tube (without caffeine and adenine). In the absence of adenine only 3 per cent of control growth

Table 1. *The influence of the concentration of conidia in the inoculum on the growth inhibition produced by caffeine.*

The extinction values, Z , were calculated according to the formula, $Z = (e_v - e_0) 10^3$, where e_0 represents the extinction of the culture immediately after inoculation, e_v the extinction after a period of incubation.

Each value represents the average of two identically arranged cultures.

Rel. conc. of conidia in inoculum	Conc. of caffeine <i>M</i>	Extinction values, <i>Z</i> , after							Growth in % of control after	
		1	2	3	4	5	6	7	5	6
		days of incubation							days of incubation	
1	—	634	1525	1626	1675	1791	1802	1692	100	100
1	7,5 × 10 ⁻³	92	168	214	241	303	411	454	17	23
1/10	—	98	1428	1737	1658	1856	1834	1704	100	100
1/10	7,5 × 10 ⁻³	—	5	15	23	30	49	62	2	3

was obtained. Adenine alone had no effect on growth even in the strongest concentrations tested.

The results confirmed the previous observations in *Neurospora* that the inhibition of growth produced by caffeine can be partly reversed by equimolar concentrations of adenine (Table 3).

If caffeine inhibits growth because it »is similar enough structurally» to metabolites of purine nature »to fit the enzyme system responsible for the metabolism of the metabolite» (Kidder and Dewey 1949), a reversal of growth inhibition is to be expected after the addition of purine metabolites, but not after the addition of compounds, which are structurally unrelated to caffeine.

In the *Neurospora* experiments it had already been found that hypoxanthine and to a lesser extent adenosine had a similar effect as adenine, a finding

Table 2. *The ability of adenine to release the caffeine inhibition of conidial growth in Ophiostoma.*

Each value represents the average of two identically arranged cultures.

Conc. of caffeine $M \times 10^{-4}$	Conc. of adenine $M \times 10^{-4}$	Extinction values, Z, after							Growth in % of control after		
		1	2	3	4	5	6	7	5 days	7 days	
days of incubation											
—	—	158	956	1481	1586	1640	1593	1677	100	100	
100	—	18	48	18	23	63	59	52	4	3	
100	1	19	42	20	55	74	76	70	5	4	
100	3	20	41	18	44	61	74	72	4	4	
100	10	6	51	26	51	74	82	80	5	5	
100	30	5	42	40	76	102	106	106	6	6	
100	100	22	71	63	93	201	277	316	12	19	
100	300	20	159	381	557	981	1115	1224	60	73	

Table 3. *The effect of various concentrations of adenine on the caffeine inhibition of hyphal growth in Neurospora crassa.*

Each value represents the average of three identically arranged cultures.

Conc. of caffeine $M \times 10^{-4}$	Conc. of adenine $M \times 10^{-4}$	Maximal hyphal length in cm after							Growth in % of control after 4 1/2 days
		1 1/2	2	2 1/2	3	3 1/2	4	4 1/2	
days of incubation									
—	—	3.7	7.3	11.1	14.8	18.6	22.4	26.1	100
80	—	1.3	3.0	5.1	7.6	10.1	12.4	14.8	56.7
80	50	2.2	4.6	7.2	9.9	12.5	15.3	18.3	70.1
80	100	2.1	4.8	7.6	10.6	13.6	16.7	20.1	70.6
80	150	1.8	4.6	7.6	10.7	14.0	17.2	20.4	78.2
80	200	2.5	5.1	8.4	11.6	14.7	17.8	21.0	80.5

which is in agreement with the antimetabolite hypothesis. In order to test the validity of this hypothesis further, a considerable number of compounds were tested for their ability to reverse the inhibition of conidial growth produced by $75 \times 10^{-4} M$ of caffeine. Some of these compounds were structurally related to caffeine, but most of them were not.

Of the compounds tested, uracil, histidine, alanine, and proline had no effect on the inhibition in concentrations up to twice that of caffeine. The vitamins, thiamine, lactoflavin and folic acid, were tested in concentrations up to 0.30×10^{-4} , 1.50×10^{-4} and $25 \times 10^{-4} M$, respectively, without obtaining any effect on the caffeine inhibition. Similarly a mixture of several vitamins in physiological concentrations was without effect. The vitamin mixture had the following composition per tube (10 ml.): inositol 115 μg , thiamine 10 μg , pyridoxine 10 μg , niacin 10 μg , folic acid 2.5 μg , vitamin B₁₂ 0.5 μg , lactoflavin 20 μg , pantothenic acid 15 μg , p-aminobenzoic acid 20 μg , and biotin 2.5 μg . 1,3,7,9-Tetramethyluric acid, theophylline, phenylalanine, and sodium salicylate proved to be inhibitors in themselves in concentrations equimolar to and/or twice that of caffeine and increased the caffeine-inhibition. In similar concentrations glycerol, glutamic acid, adipic acid, leucine, and guanosine had no effect on conidial growth of *Ophiostoma*, but nevertheless increased the caffeine-inhibition. The effect of leucine alone and in combination with caffeine appears from Figure 1.

Of the remaining three compounds tested, aniline (Figure 1) slightly inhibited growth in the absence of caffeine, whereas tryptophane and procaine were practically without effect. But in contrast to the other compounds tested aniline, tryptophane, and procaine were able to reverse the growth inhibiting effect of caffeine.

As appears from Figure 2, the antagonistic effect of tryptophane is of the same magnitude as that of adenine, whereas the other two compounds are somewhat less effective. In combination experiments the antagonistic effect

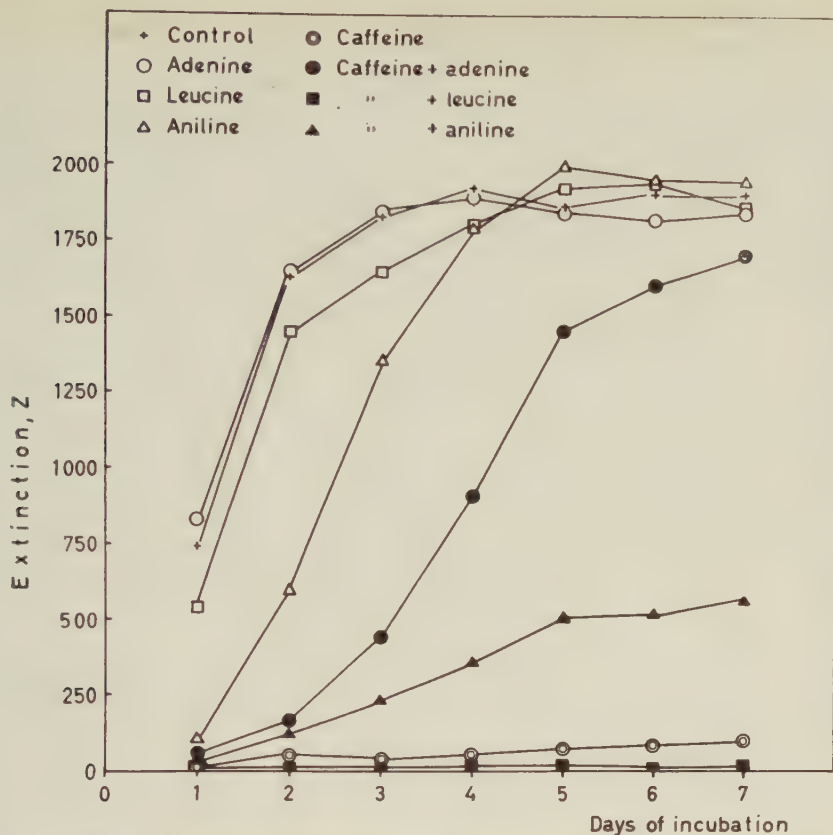


Figure 1. The effect of various compounds alone and in combination with caffeine on the conidial growth of *Ophiostoma*. Concentration of caffeine $75 \times 10^{-4} M$, of others $150 \times 10^{-4} M$.

of $75 \times 10^{-4} M$ of adenine plus $75 \times 10^{-4} M$ of tryptophane was stronger than that of the compounds alone in these concentrations, but weaker than the effect of adenine or tryptophane in concentrations of $150 \times 10^{-4} M$. A complete reversal of the caffeine-inhibition could no more be obtained with a mixture of antagonists than with the antagonists taken separately. This indicates that the antagonists, irrespective of their different structure, are acting on the same system.

The fact that procaine proved able to reverse the growth inhibiting effect of caffeine is particularly interesting, because this compound is previously known as a caffeine antagonist. If the stripped leg of a frog is immersed into a Ringer solution containing 0.2 per cent of caffeine, rigor is produced as well as a far reaching destruction of the structural elements of the muscles. However, if the Ringer solution in addition to caffeine contains procaine in

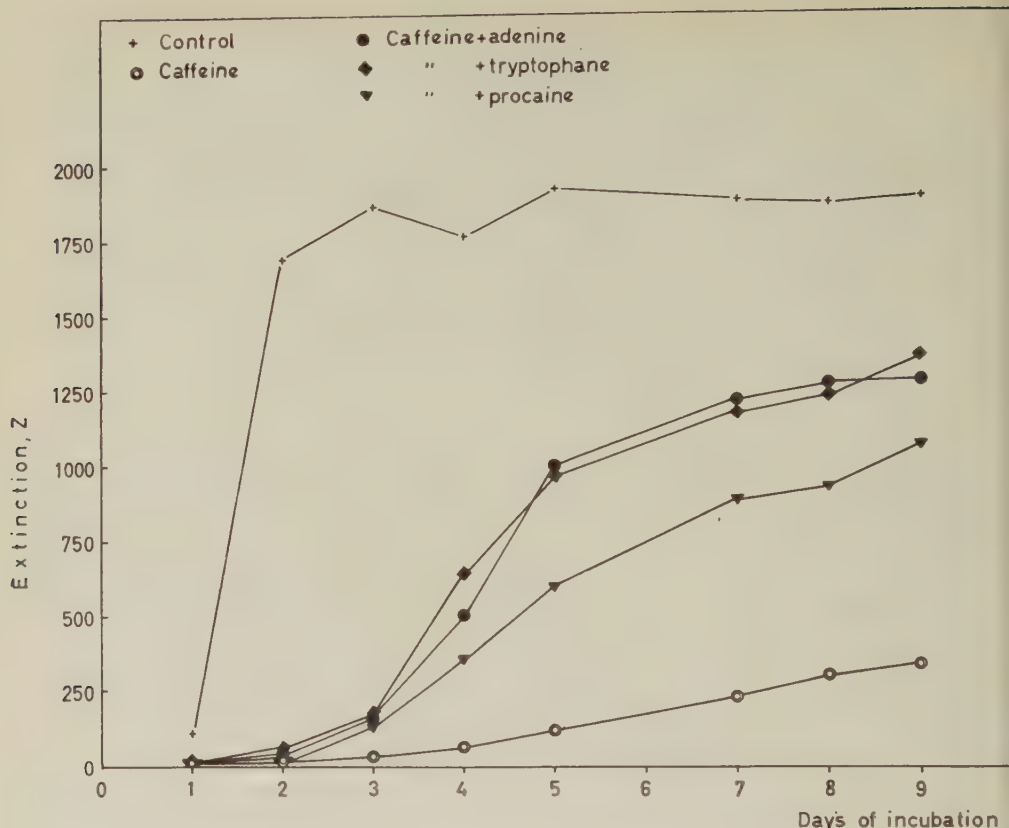


Figure 2. The influence of adenine, tryptophane and procaine on the caffeine induced inhibition of conidial growth of *Ophiostoma*. Concentration of caffeine 75×10^{-4} M, of antagonists 150×10^{-4} M.

an equimolar concentration, the condition and structure of the muscles are not altered (Schüller, 1925 b). Schüller (1925 a) explains the procaine-caffeine antagonism as due to an extracellular formation of a molecular compound between caffeine and procaine, whereas Zipf (1929) is of the opinion that procaine displaces caffeine on such intracellular surfaces — not necessarily enzyme surfaces — where the caffeine-sensitive reactions are assumed to occur.

It seems quite possible to us, that the caffeine-procaine antagonism in the case of growth inhibition is due to a similar interaction between the two compounds as that underlying the antagonism in the case of muscle rigor. On the other hand we have no reason to believe that procaine in the *Ophiostoma* experiments should act differently from the other three antago-

nists to the caffeine-inhibition of growth, viz., adenine, tryptophane, and aniline.

In any case it is clear, that whatever the explanation of the caffeine-inhibition of growth may be, nothing indicates that it should be due to a competition between caffeine and purine metabolites for purine specific enzymes. In the present investigation several compounds were found both to increase and to decrease the caffeine-inhibition. Purine compounds were represented among both types and held no unique position. (There exists, however, a similarity between the antagonists, which perhaps is significant: they are all cyclic compounds of basic and aromatic character.)

It may be concluded from our experiments, that the mere fact that the inhibition produced by a compound can be released by a structurally related metabolite, does not prove that the inhibitor is an antimetabolite before it has been shown that the metabolite in question does not share the antagonising capacity with such compounds as are structurally (and physiologically) unrelated to the inhibitor. Caution seems particularly justified when the effective concentration equals that of the inhibitor.

According to our opinion, these viewpoints may also bear upon various other cases, where purine metabolites have been found to counteract the biological effects of caffeine (e.g. Kidder and Dewey 1949, Novick and Szilard 1952). In spite of the antagonistic effects of purine metabolites, there may be other explanations of the inhibitory action of methyl xanthines than that of a competition with the essential purine metabolites at enzyme surfaces.

Summary

Caffeine in concentrations above $75 \times 10^{-4} M$ completely inhibits conidial growth of the ascomycete *Ophiostoma multiannulatum*. In agreement with previous observations in other organisms a partial reversal of this inhibition was obtained with equimolar concentrations of adenine. Since it was shown that tryptophane, aniline, and procaine are also able to counteract the caffeine inhibition, this inhibition must not necessarily be due to a competition between caffeine and purine metabolites for purine specific enzymes.

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References

- Fries, N.: Effects of different purine compounds on the growth of guanine deficient *Ophiostoma*. — *Physiol. Plant.* 2: 78, 1949.
— The production of mutations by caffeine. — *Hereditas* 36: 134, 1950.

- Kidder, G. V. & Dewey, V.: The biological activity of substituted purines. — *J. Biol. Chem.* 179: 181. 1949.
- Macht, D. I. & Schroeder, H.: Pharmakologische Studien über die Wirkung von Coffein-Adeninmischungen. — *Klin. Wochschr.* 9: 2429. 1930.
- Novick, A. & Szilard, L.: Antimutagens. — *Nature* 170: 926. 1952.
- Ryan, F. J., Beadle, G. W. & Tatum, E. L.: The tube method of measuring the growth rate of *Neurospora*. — *Amer. J. Bot.* 30: 784. 1943.
- Schüller, J.: Warum verhindern die Lokalanästhetika die Coffeinstarre des Muskels? — — *Arch. exptl. Path. Pharm.* 105: 224. 1925 a.
- Über den Antagonismus einiger Lokalanästhetika gegenüber dem Coffeineffekt am Muskel. — *Ibid.* 105: 299. 1925 b.
- Zipf, K.: Über kontrakturerregende Muskelgifte. I. Mitteilung: Beitrag zur Kenntnis des Novokainantagonismus. — *Ibid.* 140: 56. 1929.

The Gas Exchange of Flax Seeds in Relation to Temperature

I. Experiments with Immature Seeds and Capsules

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Introduction

Investigations concerning the respiration of fat-storing seeds and fruits have shown that during the period of fat formation the respiratory quotient is well above one. Gerber (5) measured the RQ-value of maturing flax seeds immediately before ripening and found the average for six seeds to be 1.22. Burr and Miller (2) in their investigation of the castor fruit made records of RQ-values from pollination to ripening. The highest respiratory quotients were found during the period of most rapid fat formation.

Determinations of RQ-values supplemented by chemical investigations have shown that fat is synthesized in the seed or fruit itself. It is now generally considered that an oxygen-rich precursor such as carbohydrate is transformed into fat. This process is supposed to be attended by the evolution of excess CO_2 , resulting in a respiratory quotient higher than one.

Although flax seeds have occasionally been employed in respiratory studies, data concerning the gas exchange before maturity are scanty. Likewise we know little about the influence of temperature on the respiratory activity of both maturing and germinating flax. Since the magnitude of the respiratory quotient to some extent depends upon fat metabolism, measurements of the gas exchange of developing flax seeds in relation to temperature may convey useful information as to the possible effect of temperature upon fat synthesis. Although conclusive results can only be obtained by a combination of physiological and chemical investigations, such measurements may give valuable

indications for further work on this subject. The respiration of immature seeds is discussed below, whereas data concerning the respiration of germinating seeds and young seedlings will be dealt with in a second paper.

Methods and Material

The apparatus described in the present paper has been called a *multithermostat*. It is a water-thermostat system which makes it possible to obtain any desired physiological temperature in six simultaneously and independently working sections for experimental periods of days or weeks. It is primarily designed for the study of gas exchange at different temperatures with Warburg instruments, but the application may be extended to many other investigations requiring a rigid temperature control.

The water-bath system is a combination of six copper containers, each with a volume of about 40 l. To obtain temperatures in the water baths below room temperature a refrigerator is installed adjacent to the baths. The refrigerator tank contains 30 per cent alcohol which is kept below 0° C by expansion coils of a 1-HP Freon compressor. A pipe system connects the refrigerator tank to the different sections of the multithermostat, the alcohol being continuously pumped through the pipes by an electric circulating pump. In each water bath the alcohol passes through copper coils immersed in the water to be chilled. Each section is supplied with an automatic thermostat system, consisting of a 70-watt heater, an adjustable contact thermometer and a mercury relay. A uniform distribution of the heat by water circulation is obtained by a stirrer. Measurements of temperature differences in the water baths showed a maximum temperature differential of 0.04° C. during testing periods of one to two hours, when readings were taken every half minute.

In order to study the gas exchange of seeds in the multithermostat, a series of modified Warburg respirometers was constructed.

A picture of the detachable respirometer flask is given in Figure 1. The cylindrical flask has a volume of about 22 ml and has neither sidearm nor centre well for alkali. For the absorption of carbon dioxide 1 ml. of 3 per cent sodium hydroxide was added to the flask. The sample to be tested was placed on a perspex disc with about one hundred perforations. The disc was held by four indentations in the glass wall. The manner of fixing the respirometers in operating position on shaking panels was adapted from an original Warburg apparatus, but continuous shaking was not applied in the experiments reported here. The reliability of this method was tested by measuring the oxygen uptake of varying numbers of immature flax seeds at a constant temperature. Samples from 10 to 40 seeds were examined and a linear relationship was obtained between oxygen uptake and number of seeds. The same result was reproduced when the seeds were placed in the bottom of the flasks and a scrap of filter paper, moistened with alkali, on the perspex disc. From these experiments it was concluded that no significant obstacle existed to the diffusion of carbon dioxide from the seeds to the absorbent when up to 40 flax seeds or an equivalent sample were used, corresponding to an oxygen uptake of about 0.4 ml. per hour.

The respirometer flasks were carefully calibrated with mercury, and the gas evolved or absorbed was calculated according to standard manometric formulae (9). The volume of the seed material was then added to the volume of the fluid phase

Figure 1. *Respirometer flask.*

of the flask system. Owing to the high water content of both immature and germinating flax seeds, the fresh weight was found to give a proper approximation to the seed volume.

In this paper the results are stated in μl . of gas absorbed or evolved per 10 seeds. As emphasized by Brown (1) the use of other standards, such as dry weight or fresh weight ought to be avoided in the case of developing seeds. Due to important changes in both fresh and dry weight during seed maturation and germination it is evident that the weight is not a satisfactory basis of comparison. When the seed number is used as a standard of reference and a uniform seed material is selected, the resulting data illustrate the development of the seed and the respiratory changes which take place, in a more satisfactory way. In the experiments with capsules, the calculated data apply to individual capsules, each normally containing 10 seeds, although the measurements were made with larger samples.

The plant material used in this study was an oil variety (Valuta) of *Linum usitatissimum*, obtained from Sveriges Utsädesförening, Svalöf. Flax plants were grown in an experimental field of oil plants at Överland, Bærum, where immature seeds were collected. In order to obtain seeds of a uniform age flowers were tagged during early blooming by woolen yarn tied around the flower pedicel. This method was used by Dillman (3) in his investigation concerning the daily growth and oil content of flax. Both in 1951 and 1952, when our experiments were performed, flowering started in the latter part of July.

At different stages of development a number of capsules of known age was collected from tagged pedicels, additional material being taken from untagged pedicels where the capsules appeared to be of the same age as the tagged ones, judged by size and colour. The seeds were then removed gently from the capsules.

Table 1. *Variations in fresh and dry weights, moisture content, and seed colour of flax seeds and capsules at different stages of development. Average values of 8 duplicate samples.*

Days after flowering 1952	Seed colour	Fresh weight		Dry weight		Moisture	
		10 seeds	1 capsule	10 seeds	1 capsule	10 seeds	1 capsule
		mg	mg	mg	mg	per cent	per cent
19	white-green	123.2	226.1	29.4	48.2	76.1	78.7
25	green	123.6	240.2	39.7	66.4	67.8	71.4
32	green, hilum yellow	123.5	230.9	47.6	74.1	61.5	67.9
41	green-yellow	119.7	222.4	63.4	80.4	47.0	63.8
46	yellow, light brown	124.4	228.8	70.2	84.9	43.6	62.9

injured seeds being rejected. A new selection based upon the colour of the seeds was performed prior to the weighing and respiration analysis. During ripening the colour changed from white to green, yellow and finally brown. (Table 1) Precautions were always taken to prevent infection of the seed material.

Samples of 10 to 30 seeds were used in the experiments, and the manometric records were started after an adaptation period in the water baths of about 2 hours. The data reported here were usually calculated from measurements made over a period of 8 hours.

Results

In the experiments carried out in 1951 six different temperatures from 0° C to 25° C were used, but in 1952 the two extreme levels were dropped. Observations of the gas exchange were made a) on flax seeds which were removed from the capsules, and b) on intact capsules detached from the pedicel.

The rates of oxygen consumption and carbon dioxide production of seeds during the first 8—10 hours after removal from the capsules are shown graphically in Figures 2 and 3. The corresponding RQ-values are given in Tables 2 and 3. An examination of these data shows that both the rate, and the value of the respiratory quotient depend on temperature. The RQ-values measured at 0° C (Table 2) approached one at three different stages of development and were distinctly different from the quotients recorded at higher temperatures. With increasing temperature the RQ-values increased up to about 1.60.

At the lower temperatures the respiratory quotient remained approximately constant during the experimental period, whereas a marked decrease was observed at 20° and 25° C. (Tables 2 and 3). In some experiments the RQ even fell below 0.8, as is also the case in fat-containing seeds during germination. The respiration curves plotted in Figures 2 and 3 show that the gradual fall of RQ at the higher temperatures was mainly related to a decrease in the carbon dioxide evolution, the changes in oxygen absorption being less pronounced.

The RQ-values observed in 1952 were somewhat higher than those from the preceding year, at corresponding stages of development. Respiratory studies of flax capsules were performed in 1952 at four different temperatures from 5° to 20° C. The observed RQ-values from the different stages of maturity are given in Table 4, and the rate of gas exchange during one of the experiments (25 days after flowering) is plotted in Figure 4. A comparison of the data in Tables 3 and 4 shows that the capsule series gave lower RQ-values. This is further illustrated in Figure 5, where the average RQ-values of seeds and of capsules from two different stages of development

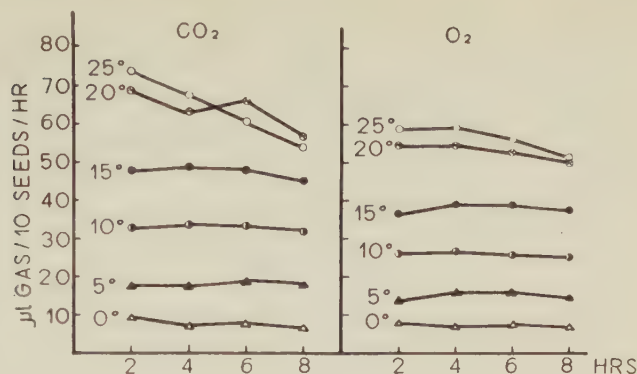


Figure 2. Respiration rate of immature flax seeds measured 19 days after flowering (1951).

have been plotted against temperature. The difference is most probably due to the respiration of the pericarp tissues having a lower RQ-value (about one) than the seeds during fat synthesis.

At an early stage of maturation (25 days after flowering) the RQ of capsules decreased significantly when the temperature was lowered from 15° to 5° C. Because of the relatively short periods of observation the decrease of RQ might possibly be ascribed to accumulation of carbon dioxide in the sappy pericarp tissue at the lower temperature. When the pericarp became woody with increasing degree of maturation, this effect probably was of minor importance. The effects of higher temperatures were similar to those described above for the seed experiments.

For a comparison of the respiratory activity at different stages of maturation, calculations were based upon the observations at 15° C. Table 5 shows

Table 2. Respiratory quotients of immature flax seeds at different temperatures and stages of maturation.

Days after flowering 1951	Experimental period hrs.	0° C.	5° C.	10° C.	15° C.	20° C.	25° C.
19	2	1.20	1.26	1.27	1.31	1.25	1.25
	4	1.03	1.13	1.26	1.25	1.16	1.15
	6	1.05	1.22	1.31	1.24	1.16	1.09
	8	1.00	1.25	1.26	1.19	1.14	1.05
27	2	0.93	1.22	1.14	1.31	1.36	1.09
	4	0.92	1.29	1.20	1.25	1.31	1.06
	6	1.00	1.37	1.13	1.21	1.26	1.02
	8	0.98	1.36	1.09	1.14	1.23	0.99
50	2	—	—	1.06	1.00	1.06	1.11
	4	1.06	0.99	1.02	0.85	0.95	0.81
	6	—	—	0.80	0.83	0.82	0.75
	8	1.03	0.98	0.94	0.77	0.81	0.71

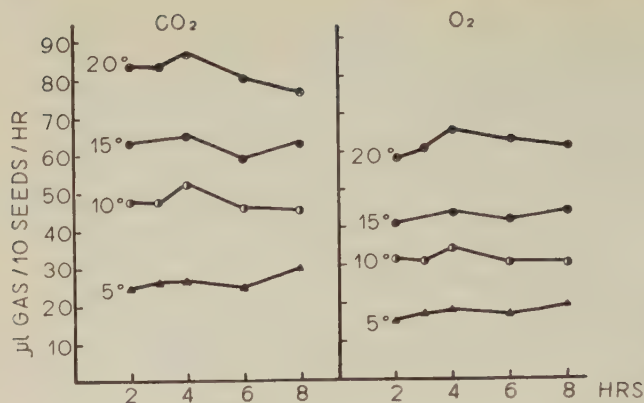


Figure 3. Respiration rate of immature flax seeds measured 25 days after flowering (1952).

both the RQ, the rate of carbon dioxide production and the excess carbon dioxide of seeds and of capsules at different stages after flowering. It appears that the RQ increased from the 19th to the 25th day, reaching a maximum value of 1.50 for the seeds and 1.31 for the capsules. During the

Table 3. Respiratory quotients of immature flax seeds at different temperatures and stages of development. Average values of 3 duplicate samples.

Days after flowering 1952	Experimental period hrs.	5° C.	10° C.	15° C.	20° C.
19	2	1.22	1.26	1.31	1.35
	3	1.21	1.27	—	—
	4	1.22	1.22	1.29	1.29
	6	1.18	1.23	1.27	1.30
25	2	1.51	1.51	1.55	1.43
	3	1.52	1.52	—	1.33
	4	1.43	1.50	1.44	1.32
	6	1.50	1.49	1.41	1.27
32	8	1.51	1.46	1.42	1.25
	2	1.32	1.53	1.36	1.55
	3	1.34	1.60	1.43	1.50
	4	1.36	1.52	1.40	1.45
41	6	1.31	1.49	1.39	1.30
	8	1.41	1.47	1.39	1.34
	2	1.37	1.58	1.57	1.46
	3	1.43	1.36	1.52	1.40
46	4	1.32	1.47	1.55	1.33
	6	1.34	1.45	1.50	1.24
	8	1.40	1.40	1.36	—
	2	1.29	1.41	1.25	1.12
46	3	1.28	1.28	1.32	1.05
	4	1.29	1.39	1.23	0.99
	6	1.30	1.38	1.22	0.95
	8	1.29	1.36	1.20	0.90

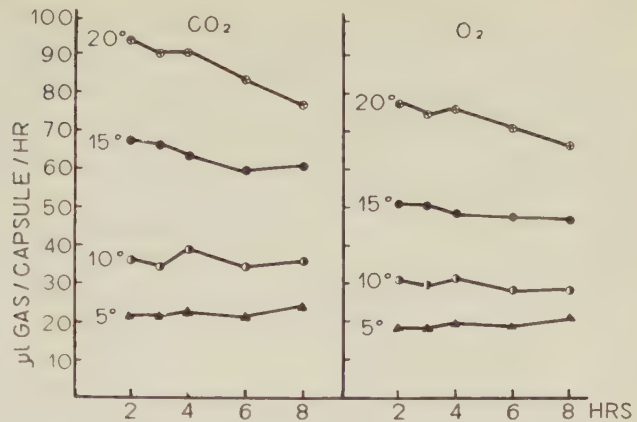


Figure 4. *Respiration rate of immature flax capsules measured 25 days after flowering (1952).*

subsequent 16 days only slight deviations from these values were observed, but 46 days after flowering the RQ of both seeds and capsules decreased significantly. The changes in carbon dioxide production did not correspond to the RQ variations. A maximum was observed 25 days after flowering, and

Table 4. *Respiratory quotients of flax capsules at different temperatures and stages of development.*

Days after flowering 1952	Experimental period hrs.	5° C.	10° C.	15° C.	20° C.
19	2	1.03	1.00	1.16	1.10
	4	1.02	1.06	—	—
	6	1.03	1.03	1.16	1.08
	8	1.03	1.04	1.13	1.09
25	2	1.16	1.16	1.32	1.26
	3	1.17	1.17	1.32	1.22
	4	1.12	1.24	1.30	1.20
	6	1.13	1.22	1.26	1.18
	8	1.16	1.24	1.29	1.16
32	2	1.12	1.34	1.24	1.20
	3	1.13	—	1.22	1.21
	4	1.13	1.30	1.32	1.25
	6	1.11	1.26	1.27	1.22
	8	1.10	1.33	1.23	1.20
41	2	1.31	1.48	1.35	1.29
	3	1.23	1.38	1.33	1.24
	4	1.25	1.41	1.26	1.19
	6	1.27	1.32	1.21	1.15
	8	—	1.23	1.19	1.10
46	2	1.27	1.25	1.17	1.17
	3	1.21	1.20	1.18	1.18
	4	1.19	1.16	1.15	1.11
	6	1.13	1.13	1.09	1.12
	8	1.14	1.10	1.07	1.05

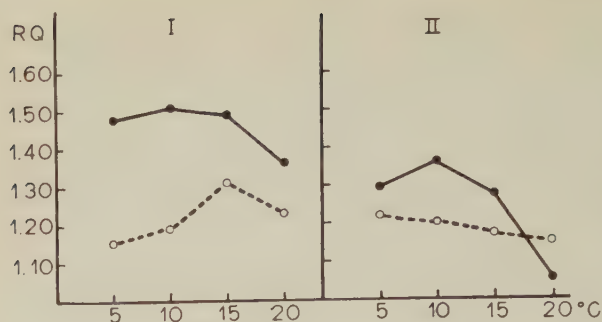


Figure 5. *Respiratory quotients of flax seeds and capsules at different temperatures. Average values for the first 4 hours of observation. Fully drawn lines: seeds, broken lines: capsules. I: 25 days after flowering, II: 46 days after flowering.*

Table 5. *Respiratory drift of flax seeds and capsules during development. Average values from the first 4 hours of observation. CO₂ output and excess CO₂ given as μ l per hour per 10 seeds or per capsule. Temperature 15° C.*

Days after flowering 1952	Seeds			Capsules		
	RQ	CO ₂ output	Excess CO ₂	RQ	CO ₂ output	Excess CO ₂
19	1.30	49	11	1.16	51	7
25	1.50	64	21	1.31	66	16
32	1.40	56	16	1.26	60	12
41	1.55	32	11	1.31	48	11
46	1.27	21	5	1.17	39	6

then the rate fell gradually. From Table 1 it will be seen that 46 days after flowering the seeds were still unripe and had a high water content, and that the dry weight was still increasing.

Discussion

The significance of the respiratory quotient is not quite evident in the case of maturing fat-storing seeds. It seems to be fairly well established that essentially fat synthesis takes place by condensation reactions between intermediates in carbohydrate breakdown, coupled with complete oxydation of such intermediates through the Krebs cycle. Hence, factors which affect the quantitative balance between condensation and combustion must have a corresponding influence on the magnitude of the respiratory quotient. For this reason it is not possible to estimate a definite theoretical RQ-value, as in the case of germinating seeds.

An important feature of the present results is the low respiratory quotients which were observed at 0° C., indicating a strong depression of fat formation and a predominance of sugar respiration at this temperature. The pronounced

increase of RQ when the temperature is raised to 5° C. indicates a stimulation of the fat synthesis, the change being too great to be caused by changes in the accumulation of gases in the tissues. Apparently, therefore, the minimum temperature of fat synthesis in flax seeds lies somewhere between 0° and 5° C.

The highest RQ-values of both seeds and capsules were observed at the intermediate temperatures, and accordingly it might be suggested that an optimum temperature for fat synthesis exists somewhere between 10° and 20° C. However, the lowering of RQ at higher temperatures need not necessarily indicate a decrease in the rate of the synthetic process per se, because other temperature effects may interfere. In particular the possibility must be taken into account that a depletion of the carbohydrate reserves of the seeds results in fat respiration. The falling-off of the respiratory quotients after a few hours at the higher temperatures, which is apparent from our diagrams and tables, indicate a comparatively rapid exhaustion of these reserves. We know that fat combustion takes place during normal germination of flax, and Dillman (3) has demonstrated that even immature flax seeds may show a high percentage of germination when placed on moist filter paper. It is likely that the oxidation of fat does not start until most of the carbohydrates have been consumed. The sugar content of flax seeds during maturation has been studied by Ivanov (6) who found that at an early stage the total amount of sugars did not exceed 5 % of the dry weight. Later the sugar content decreased. From the present data it may be calculated that at 25° C a sugar content of 5 % would last for about 15 hours of respiration, neglecting the simultaneous fat synthesis.

Thus it seems probable that fat synthesis continued for some hours after the seeds or capsules were detached from the plant and was then substituted by fat combustion. The high RQ-values observed for several hours at intermediate temperatures may be considered to indicate an *economic* optimum temperature for fat production possibly at about 15° C.

It has been reported that a temperate or cold climate is more favourable for the formation of unsaturated fatty acids than a tropical one. Thus, it has been observed that flax cultivated in northern regions yields an oil with a higher iodine number than oil from more tropic regions. (Ivanov 7, Painter et al. 8) This difference is supposed to be related to the temperature, although a satisfactory explanation of the possible effect of temperature upon the unsaturation of fatty acids is lacking.

It might be suspected that a fat respiration, similar to that indicated by the present results, may occasionally occur in the field if the sugars of the immature seeds happen to be temporarily exhausted. This might particularly occur when flax is cultivated in a warm climate with a short daylength, where the respiration loss is high. If the unsaturated acids are preferentially

utilized, as they apparently are during germination (6), this would lower the degree of unsaturation, giving the oil a lower iodine number than oils from a colder climate.

Studies concerning the RQ-changes during maturation of fat-storing seeds have shown that the highest RQ-values are observed during the period of most rapid fat formation (2). Many authors have reported that the oil formation proceeds at an extraordinarily rapid rate for a short period during the development of the fruit or seed (4). In the present experiments the highest RQ-values were observed between the 25th and the 41st day after flowering, this interval apparently indicating the period of most rapid fat formation (Table 5). However, it is obvious that the respiratory quotient alone is not an adequate measure of the intensity of fat formation. If the fat synthesis from the carbohydrates is attended by the evolution of excess CO_2 , it is evident that the difference between carbon dioxide evolution and oxygen consumption has to be considered also. Strong fat formation may be assumed to depend upon a rapid rate of respiration. From Table 5 it may then be concluded that there was a maximum intensity of fat synthesis about 25 days after flowering. At that time the seeds were green and had a high water content. The unchanged RQ-values but lowered respiratory activity 41 days after flowering suggest that carbohydrates were still converted into fats, but that both sugar oxidation and fat synthesis were markedly retarded.

Summary

(1) — An apparatus for measuring the gas exchange of plant material at different temperatures was constructed.

(2) — The oxygen consumption and carbon dioxide production of detached immature flax seeds and capsules were examined at temperature levels between 0° and 25° C at different stages of development.

(3) — At 0° C the RQ-values of seeds were found to be near one, suggesting that fat synthesis was strongly restricted at this temperature. At the other temperatures RQ reached values well above one. The quotients were higher for seeds than for capsules.

(4) — A constant respiratory activity for several hours was maintained at intermediate and lower temperatures only. At higher temperatures the RQ-values normally decreased after a few hours, apparently due to exhaustion of carbohydrates and a change to fat respiration.

(5) — Developmental changes in the RQ-values were less pronounced than the changes in respiration intensity. The period of the highest respiratory activity is supposed to coincide with the period of most rapid fat formation.

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References

1. Brown, R.: The Gaseous Exchange of Seeds and Isolated Cotyledons of *Cucurbita Pepo*. — *Ann. Bot.* 6: 293. 1942.
2. Burr, G. & Miller, E.: Synthesis of Fats by Green Plants. — *Bot. Gaz.* 99: 773. 1937.
3. Dillman, A. C.: Daily Growth and Oil Content of Flax Seeds. — *J. Agr. Res.* 37: 357. 1928.
4. Eyre, J. V.: Notes on oil development in the seed of a growing plant. — *Biochem. Journ.* 25: 1902. 1931.
5. Gerber, C.: Etude comparee de la respiration des graines oleagineuses pendant leur developpement et pendant leur germination. — *Actes du Congres Internat. de Botanique*, Paris. 59—101. 1900.
6. Ivanov, S.: Über den Stoffwechsel beim Reifen ölhaltiger Samen mit besonderer Berücksichtigung der Ölbildungsprozesse. — *Beihefte Bot. Centralbl.* 28: 159. 1912.
7. — The factors in the process of oil formation in plants. — *Österr. Chem. Zeit.* 32: 89. 1929.
8. Painter, E. P., Nesbitt, L. L. & Stoa, T. E.: The influence of seasonal conditions on oil formation and changes in the iodine number during growth of flaxseeds. — *Amer. Soc. Agr. J.* 36: 204. 1944.
9. Umbreit, W.W., Burris, R. H. & Stauffer, J. F.: *Manometric Techniques and Related Methods for the Study of Tissue Metabolism*. — Burgess Pub. CO. Minn. 1948.

On the Nature of Photoperiodic Induction

III. The Summation of the Effects of Inductive Photoperiodic Cycles

By

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It is clear from what has been reported in the first two papers of this series (Carr, 1953, I & II) that summation of the effects of inductive photoperiodic treatments applied to the leaves of herbaceous plants takes place in the shoot apex. Considerable interest, therefore, attaches to the persistence within the shoot apical meristem of the effects of photoperiodic treatments which are not in themselves sufficient to bring about the transition to flowering, i.e. sub-threshold photoperiodic stimuli, and to the effects of non-inductive conditions on the survival of such stimuli. For certain long-day and short-day plants it has been shown that the effects of non-consecutive photoperiodic treatments may be summated, and that interpolated non-inductive cycles have little or no effect on summation. Lang (1952) denies the possibility of summation in short-day plants, and states that «fractional induction is only possible in long-day plants», but this generalisation, as will be seen from the review and experimental results here reported, is too sweeping. On the other hand, summation appears to be possible only when some irreversible stage of induction has been reached, or when the number of interpolated non-inductive cycles is small enough to permit sub-threshold inductive effects to persist.

Summation in long-day plants

It has been shown by Naylor (1941) that groups of three consecutive long-day cycles separated by groups of three consecutive short-day cycles are not summated

in annual beet and do not cause flowering. In Hamner's (1940) experiments on the same plant, long-day treatments were given in groups of 10 consecutive cycles and summation took place, even when these periods of induction were separated by 16 short days. According to Naylor, 13 consecutive long days are required for the induction of flowering in annual beet. In that case, a single group of 10 inductive cycles is probably sufficient to bring about an irreversible, but sub-threshold, change, and even two or three inductive cycles given at a later date may sufficiently augment this change to such a degree that flowering will take place. In Naylor's experiments flowering did not occur with repetition of a regime consisting of a long day or 24 hours of light, followed by a short day or 24 hours of darkness. In annual beet the inductive effect of a long day is, therefore, completely lost in a succeeding long dark period or short day. Lang and Melchers (1943) have shown that 3 days of continuous light produce an irreversible induction in annual *Hyoscyamus niger*, and that this period is the minimum required for flower initiation. Interpolation of up to 10 short days between successive groups of 3 long days does not prevent summation. Rupcheva (1948 b) claims that the flowering of *Anethum graveolens* and spinach, both long-day plants, is not affected very much by alternation of short days with long days, but that the flowering of other long-day plants (oats, *Carthamus tinctorius*) is markedly postponed. Garner and Allard (1913) found that the long-day plant, *Impatiens Balsaminea*, flowers as rapidly with alternating short-days (10 hour photoperiods) and natural long days, as with consecutive long days. Samygin (1948 b), working with *Rudbeckia bicolor*, used light of different intensities in the intercalated short days. With light of 7 ft. candles during the 10-hour short photoperiods, flowering was not much delayed with alternations of one long day and either one or two short days, as compared with controls in long days. There was a considerable delay with sunlight during the short photoperiods, and no flowering occurred with single long days alternating with 2 short days at this high light intensity. Nor did flowering occur under the short days alone at either light intensity, showing that the short photoperiods used were, in fact, non-inductive for this plant. This suggests that the inhibitory effect of intercalated short days (with a long-day plant) is connected with high light intensity during the photoperiod. Melchers and Lang (1948) reached the opposite conclusion, namely, that interruption of induction must be the more inhibitory the longer the dark periods in the intercalated cycles. This is not compatible with the results of Claes and Lang's experiments, in which summation took place, in *Hyoscyamus niger*, of consecutive cycles of 7 hours of light and 41 hours of darkness.

It is of interest in this connection that in the long-day plant, *Urtica pilulifera*, leaves kept in the dark or in short days with 8- or 10-hour photoperiods are antagonistic to the action of leaves kept in long days, but leaves kept in short days with from 1- to 3-hour photoperiods are not antagonistic (Lona, 1947 b). The suggestion may be made that the decline in induction which takes place in non-inductive cycles following an inductive cycle is in some way connected with the production of some substance antagonistic to flowering, which is not formed in photoperiods of low light intensity or of very short duration.

Summation in short-day plants

Garner and Allard (1931) carried out some long-term experiments using short-day plants, treating them with alternate short days (10-hour photoperiods) and long days

(natural daylength of the summer, in Washington). In all cases flowering was greatly hastened as compared with controls under natural daylength throughout. For instance, *Perilla ocyroides* flowered in 55 days as compared with 84 days for the controls; *Cosmos* flowered in 68 days as compared with 113 days, and Biloxi soybean flowered in 42 days as compared with 72 days. In Long's (1939) experiments on Biloxi soybean the plants did not flower with one or with two consecutive short days, but all plants treated with 4 or more consecutive short days produced some flower primordia. The experiments appear to have been conducted with very young plants. Occasionally, older plants flowered with only two short days. As a result of experiments in which one or two short days were given, followed by a number (up to 20) of long days, and then a further number (up to 5) of short days, Long concluded that the effects of one or of two short days do not persist over even as few as 5 long days. In another experiment in which two short days were given, followed by $10 \times (1+1)$, $10 \times (1+2)$ etc. up to $10 \times (1+5)$ treatments, i.e. in each case 10 single short days with up to 5 long days in between each short day, no flowering was obtained. Thus, the effect of a single short day did not persist over a period of 24 hours. Finally, Long used treatments in which induction by 6 short days was followed, after 10 or 20 long days, by a further 1 to 9 short days. In this experiment all his plants flowered, and the total number of flowers per plant was counted. It was found that the number of flower primordia produced as a result of two separate treatments with short days was equal to the sum of the numbers produced by each treatment separately. Thus, in Biloxi soybean, two inductive treatments with more than 4 consecutive short days act independently and additively if separated by one or more long days, but the products of sub-threshold stimuli (less than 4 consecutive short days, in Long's experiments) are not summated into effective stimuli. According to Hausschild (quoted by Lang, 1949) alternations of short and long days do not produce flowering in *Kalanchoe Blossfeldiana*, and even with alternations of 2 short days with one long day there is some delay as compared with plants in continuous short days. Westphal (also quoted by Lang) found that there was some delay with alternations of 4 short and 6 long days, whereas alternations of 7 short and 7 long days, or 14 short and 14 long days resulted in more rapid flowering than continuous shortday treatment. It can also be deduced from data given by Harder and Gümmer (1949) that flowering does not occur in *Kalanchoe* with alternations of single short days and single long days. Rupcheva's (1948b) experiments on *Perilla* confirmed Garner and Allard's result, that flowering does take place, although with some delay, in a regime of alternating long and short days, and also in a regime of alternations of 2 short days and 2 long days. No flowering occurred in alternations of 3 short days and 3 days of continuous light, or 2 short days and 2 days of continuous light, but there was good summation in alternations of 3 short days and 3 days of continuous darkness. *Chrysanthemum* behaved in a similar manner. Potapenko (1947) found that in *Bidens tripartita* summation was quite effective with alternations of $(1+1)$, $(1+2)$ and $(1+3)$, where the first figure indicates a short day, the second figure a number of intercalated long days (17-hour photoperiods) (Figure 1).

Post experimented on the Goldsmith variety of *Chrysanthemum morifolium* and found (1950) that 4 consecutive short-day cycles are sufficient to induce flowering, but that if a long day is intercalated between the last two short-day cycles flowering does not occur. This may be interpreted to mean that four consecutive cycles are required for threshold induction, and that the effect of three cycles is sufficiently diminished by a single long day to prevent the attainment of the threshold. Samygin

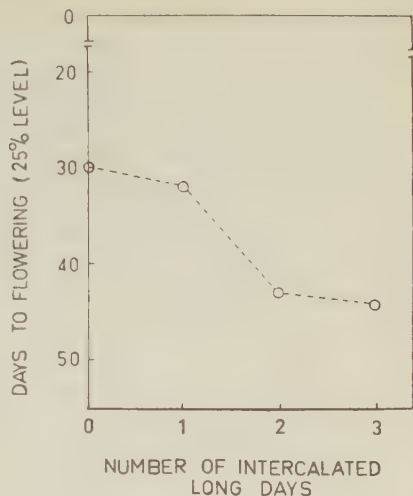


Figure 1. *Bidens tripartitus*. Summation of inductive effects of single short days, according to data from Potapenko, 1947. Note: the curve in this and those in the other figures indicate trends but not intermediate values, since fractions of long days are inadmissible.

(1948b) found that summation in alternations of (1+1) and (1+2) (notation as above) with *Perilla* is not affected by the light intensity during the non-inductive photoperiods. This is in contrast with the light intensity effects found with long-day plants.

Summation is to be expected in *Xanthium pennsylvanicum*, since each separate short-day cycle produces an inductive effect which is above the threshold value. Summation in alternations of (1+2) has been tested by Roberts (1951), and it has been found that there is little increase in certain flowering responses when more than 4 repetitions of such treatment are applied (i.e. a total of 4 short days). By depressing the temperature in the dark period to 4.4° C. Long (1939) found that 8 consecutive short days are then required for induction of flowering in *Xanthium*. This enables summation to be tested more easily, since each short-day cycle is then well below the threshold for induction. The results of Long's experiments show that the residual effect of 4 consecutive long dark periods at 4.4° C. persists over a long photoperiod at 21° C., but not over two such long photoperiods. Moreover, it is impossible to obtain flowering by extending the length of a single dark period at 4.4° C. — periods of darkness up to 112 hours gave no flowering.

Summation, then, is possible in both long-day and short-day plants. It is more effective in some plants than in others, but this is independent of the photoperiodic classification. It appears that the product of an inductive cycle, unless it reaches some threshold value and is conserved, may decay at a rate which is determined largely by the plant species, but is also affected by light and darkness, and probably also by temperature. Decay is favoured by conditions which may be expected to be optimally non-inductive, i.e. long photoperiods with short-day plants, sub-critical photoperiods at high light intensities with long-day plants.

Experimental work

From the review given above it may be seen that there have been few *comparative* experiments on summation in long-day and short-day plants, and that the ranges of treatments over which summation has been studied have generally been too narrow. The experiments reported below were carried out at the Melchers Abteilung of the Max-Planck Institut für Biologie in Tübingen, at the suggestion of Professor Melchers. Seeds of Biloxi soybean were obtained through the courtesy of Dr. H. A. Borthwick, U.S. Department of Agriculture.

Experiment 1. *Hyoscyamus niger*.

The previous work on this long-day plant (Lang and Melchers, 1943) may be summarised as follows:

- (a) flowering does not follow after treatment with 2 long days;
- (b) the stimulus generated by 2 long days is retained in the plant for up to 10 days (short days) and can be added to another such sub-threshold stimulus so as to promote flowering;
- (c) the length of the light period in the intercalated short days has little effect on the permanence of the stimulus generated by 2 long days;
- (d) summation of stimuli resulting from *single* long days was not investigated, nor was summation of stimuli resulting from pairs of long days with more than 10 intercalated short days; nevertheless, the conclusion was drawn that some stable, summatable substance is formed as a result of the action of a long day.

In the experiment, two sets of plants were used, set A from seeds sown on 29th November, 1951, and set B from seeds sown on 17th April, 1952. The older plants were more vigorous and had larger rootstocks than the younger. The experimental treatments were begun on 11th June, 1952. Since it was desired to estimate the numbers of leaves formed from the beginning of the treatments until the onset of flowering, a number of plants of each set were dissected at the outset, and their leaves, including primordia, removed and counted. These counts gave the following results:

Set A 10 plants.	Leaf number 38.2 ± 2.15^1
Set B 12 plants.	Leaf number 31.5 ± 1.75^1

From the beginning of the experimental treatments records were kept of senescent leaves removed from the plants, and the increases in leaf number were determined by subtracting the respective starting number from the counts made when the plants were dissected, adding, of course, the number of senescent leaves removed.

Photoperiods were given using daylight in a south-facing greenhouse, the temperature of which was kept between 20 and 25° C. Each day the plants were brought into the greenhouse from the darkroom, which was kept at 21° C., at 7.30 a.m. Groups of plants receiving a short day were returned to the darkroom at 4.30 p.m. and groups receiving a long day at 11.30 p.m. Thus, in short days the plants received 9 hours

¹ Standard deviations of the mean.

of daylight, and in long days 16 hours of light, consisting mainly of daylight with between 2 and 4 hours of supplementary illumination from filament lamps giving an intensity at plant level of 280 ft. candles. Before the experimental treatments were begun the plants had been cultivated since sowing in short days (9 hours).

The following terminology will be used to describe the experimental treatments: $6 \times (1+1)$ indicates that six long days were given, with a short day in between each long day. Similarly, $3 \times (2+2)$ indicates that three pairs of long days were given with two short days in between each pair of long days. The treatments used were as follows: $6 \times (1+1)$, $4 \times (1+2)$, $6 \times (1+12)$, $3 \times (2+1)$, $3 \times (2+2)$, $3 \times (2+12)$. In addition, other groups of plants were treated with one, two or six long days, and a control group was kept throughout in short days. At the end of the experiment the group which had received one long day was given another two long days, and the group which had had two long days was treated with another long day, so as to bring the total of long days received up to three. Towards the end of the experiment, when four of the ten groups were still left, the others having been already dissected and found to be flowering, some of the plants were attacked by mites, which eat the contents of the rootstock but do not attack the growing point. In many cases these plants had to be dissected prematurely, and the number of plants in some groups at the end of the experiment was too small to be regarded as satisfactory. Plants which were either not attacked during this period or which survived the attack remained vigorous until the end of the experiment. The results are presented in Table 1.

From these results it can be seen that all plants of all groups, with the exception of A (short-day controls), B, C and G, flowered as a result of the experimental treatments. One plant of group B, none of group C and only 4 of the 14 plants in group G flowered. It is clear, therefore, that, within the conditions of the experiment, the stimulus generated by a long day is partially, if not completely, lost in an ensuing long period of short days. Lang and Melchers (1943) have shown that not less than three consecutive long days are required for induction of *Hyoscyamus niger*, and that induction is full with six consecutive long days. If the effect of a long day or of two consecutive long days remains permanent in the plant it is difficult to explain why at least some of the plants of, e.g., group K should not have flowered immediately after having received only four of the six long days applied. The only available explanation is that the effect of induction by sub-threshold stimuli is gradually weakened in succeeding non-inductive photoperiodic cycles. If we could measure the amount of delay caused by the intercalated short days we could obtain an estimate of this weakening or decay of the stimulus. Such an estimate can be obtained if we are permitted to make the assumption that the leaf-plastochron interval is not directly affected by sub-threshold stimuli. It can be calculated from the increases in leaf number over given times that a new leaf was formed every two days, in both sets of plants. We can, therefore, calculate the number of leaves formed up to the end of the period of *induction* (i.e. after the last long day). Any excess of leaves over and above this number will, therefore, represent the number of leaves formed

Table 2. Estimation of the delay in flower formation following the last long day cycle of the treatments listed in Table 1 (*Hyoscyamus niger*).

Group	Total number of days in induction period (long days plus intercalated short days)		Increase in leaf number from beginning of induction		Delay in formation of flower primordia ($= 2 \times [b-a]$) to nearest day
			Calculated	Observed	
		Days	(a)	(b)	Days
Set A	D	6	3	5.1	4
	E	11	5.5	5.5	0
	F	10	5	6.6	3
	G	66	33	(40)*	(14)
	H	8	4	7.8	7
	J	10	5	6.5	3
	K	30	15	16.5	3
Set B	D	6	3	6.4	7
	E	11	5.5	7.25	3
	F	10	5	8.0	6
	G	66	33	(44)**	(22)
	H	8	4	7.1	6
	J	10	5	8.0	6
	K	30	15	15.9	2

* 2 plants only.

** 1 plant only.

after the completion of the inductive treatment. Now it is commonly found that in photoperiodic experiments the delay in production of flower primordia is in proportion to the »strength» of the photoperiodic stimulus. By estimating the differences between the (calculated) number of leaves formed in each group during the inductive treatment and the *actual* number preceding flower initiation, it should be possible to demonstrate the weakening of the photoperiodic stimulus in non-inductive photoperiodic cycles. The data for group F must be considered separately, since these plants received only 4 long days, and the delaying effect of intercalated short days might be different from that in other groups which received 6 long days. In view of the small number of plants used, and the large standard deviations of the means of leaf numbers no significance can be attached to the differences between groups D, E, H and J (Table 2).

There is, however, a striking difference between the values for $6 \times (1+12)$ and $3 \times (2+12)$ in both sets of plants. In the latter group flowering took place with a delay of only two or three days after the last of the 6 long days; this means that the inductive stimulus of paired long days is effectively summated. The fact that the delay is somewhat longer in the control group with six consecutive long days may be attributed to the possibility that induction may sometimes be completed after 4 long days. Thus, it is possible

Table 3. *Summation of photoperiodic induction in Chenopodium amaranticolor in various regimes of short and long days.*

Group (10 plants in each)	Treatment	Total number of short days given	Days to flowerformation
A	L.D. controls.....	0	—
B	1 S.D. + 1 L.D.....	8	17
C	1 S.D. + 2 L.D.....	9	26
D	1 S.D. + 4 L.D.....	7	34
E	2 S.D. + 1 L.D.....	10	15
F	2 S.D. + 2 L.D.....	8	16
G	2 S.D. + 4 L.D.....	6	19
H	5 S.D. + 1 L.D.....	10	12
J	5 S.D. + 2 L.D.....	10	14
K	5 S.D. + 4 L.D.....	10	15

that the low value of the delay with $3 \times (2+12)$ conceals a larger delay following the second pair of long days, i.e. this group might have flowered without the intervention of the last pair of long days, but with some considerable delay. This requires further testing. Examination of the original data of Lang and Melchers (1943) on the basis adopted above shows that the delay following induction was in all cases far smaller than in the present experiment, and the values are consistent with the possibility that induction had actually been completed after the fourth long day. It is interesting to note that in their 1942 experiments the conditions were such that 4 out of 5 plants flowered after treatment with 4 long days (of 16 or 24 hours of light), and that the delay as measured by the increment in leaf number was no greater with 4 long days than with 10 long days. Returning to the discussion of the present experiment we find that the delay with $6 \times (1+12)$ was considerable (14 and 22 days respectively in sets A and B). Indeed it is possible that had the plants of this group been kept longer after the end of the treatments, more would have been found to be flowering (a period of 20 days elapsed between the last long day and the date of dissection). However, this does not obviate the main conclusion, that the photoperiodic stimulus generated by a long day becomes dissipated during succeeding short days and is probably eventually lost (c.f. groups B and C). This conclusion is contrary to that reached by Lang and Melchers and implies that the substance formed in a *single* long-day cycle is not permanent and stable within the plant.

Experiment 2. *Chenopodium amaranticolor* Coste et Reyn.

This plant is said by Lona (1950 c) to require only one short day for induction. In a preliminary experiment plants, which were very uniform in appearance, from seeds of Lona's strain sown on 17th April, 1952 were treated with from one to five short days (9 hours of light per day) beginning on 11th June. At this time the plants each had some 11 fully grown leaves, and were fairly uniform in height, ranging

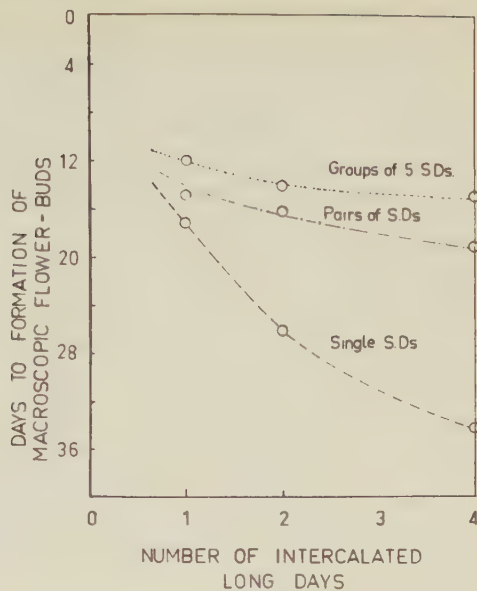


Figure 2. *Chenopodium amaranticolor*, Coste et Reyn. Summation of inductive effects of single short days, or of groups of consecutive short days.

around a mean of 35 cm. Plants treated with short days showed an immediate stimulation of height growth, and those treated with 5 or 4 short days were classified as flowering on 9th July. Those treated with 2 short days were similarly classified on 16th July. The long day controls and the plants treated with one short day were grown on until 4th September. Although the plants of these groups were then practically identical in height (182 cm.), the plants treated with one short day were more branched; on dissection, no indication of flowering was found in either group. For our purposes then, this strain of *Chenopodium amaranticolor* does not flower when treated with a single short day.

Ten groups of plants, ten per group from seeds sown on 17th April, were used for the summation experiment. The conditions were the same as those described above for *Hyoscyamus*, and the treatments were begun on 29th May, when all groups, with the exception of the long-day controls, received a short day. The treatments used were: (1+1), (1+2), (1+4); (2+1), (2+2), (2+4); (5+1), (5+2), (5+4), where the first number indicates short day(s) and the second, long day(s). These treatments were repeated until the plants reached a subjectively determined stage in flowering (appearance of a macroscopic terminal inflorescence). The terminal buds of the plants were then dissected to verify the presence of flower primordia. The results are given in Table 3 and Figure 2.

All groups, with the exception of the long-day controls, flowered within 35 days of commencing treatment. This means that in groups B, C and D the stimulus generated by single short days is summated. From Figure 2, however, it can be seen that the delay in flowering, which is small when *pairs* of short days are given, is considerable when *single* short days are separated by 2 or by 4 long days.

Table 4. *Summation of photoperiodic induction in Biloxi soybean in various regimes of short and long days.*

Group	Treatment	Number of plants	Number of plants flowering	Number of flower primordia per plant (mean with standard deviation of mean)
A	L.D. controls	10	0	0
B	1 S.D.	10	0	0
C	2 S.D.	10	10	7.6 \pm 3.99
D	6 S.D.	10	10	105 \pm 16.9
E	10 S.D.	10	10	132 \pm 13.8
F	10 \times (1 S.D. + 1 L.D.) ...	10	2	2
G	10 \times (1 S.D. + 2 L.D.) ...	10	0	0
H*	10 \times (1 S.D. + 12 L.D.) ...	10	(0)	(0)
J	5 \times (2 S.D. + 1 L.D.) ...	10	10	43.6 \pm 7.48
K	5 \times (2 S.D. + 2 L.D.) ...	10	10	34.4 \pm 8.32
L	5 \times (2 S.D. + 12 L.D.) ...	10	10	46.2 \pm 14.74

Analysis of variance test between groups J, K and L gives $(F) = 1.56$ with a probability of these groups belonging to different populations of 1 %.

* Plants received only 6 \times (1 SD + 12 L.D.) at the conclusion of the experiment.

It is interesting that the most rapid »decay» (delay in flowering) takes place with the first intercalated long day; after that the intercalation of further long days has a relatively smaller effect. The rate of breakdown of the product of the short-day cycle appears to be proportional to its concentration. This product must, however, become stabilised in short days, since the rate of fall of the curves becomes less with increase in the number of consecutive short days.

Experiment 3. *Biloxi soybean.*

Eleven groups of plants, 10 per group, were used for the experiment; these plants were selected for uniformity from among 400 plants grown from seeds sown on 8th June, 1952. Each plant had two fully expanded trifoliolate leaves at the beginning of the experimental period. The conditions were the same as those for experiments 1 and 2, except that towards the end of the experiment plants receiving long days were allowed to stand in the greenhouse under supplementary illumination, instead of being removed to the darkroom at 11.30 p.m. The treatments were as follows: 10 \times (1+1), 10 \times (1+2), 10 \times (1+12), 5 \times (2+1), 5 \times (2+2), 5 \times (2+12), the notation being as for *Chenopodium*. Other groups received 1, 2, 6 or 10 consecutive short days. It was arranged that these groups should receive their short-day treatments in the middle of the experimental period. The plants of each group were dissected one month after having received the last short day, and the number of flower primordia counted. As a result of the data collected during the early part of the experiment it was seen that flowering could not be expected to take place in the group receiving 10 \times (1+12). Treatments in this group were, therefore, stopped when only 6 \times (1+12) had been applied. Apart from this, these plants had by that time become too large for convenient experimentation. The results are given in Table 4, and in Figure 3.

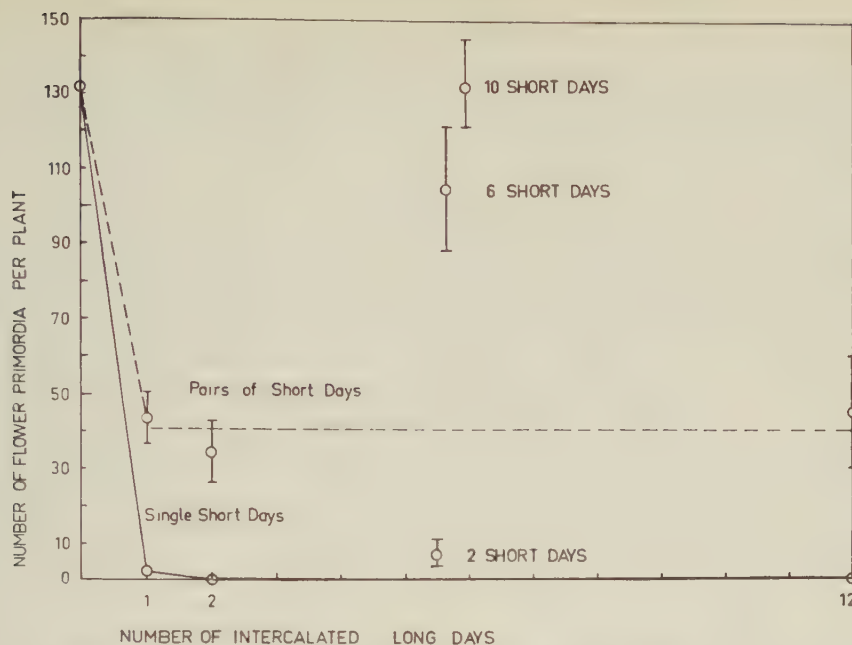


Figure 3. *Biloxi soybean*. Summation of photoperiodic stimuli. The data of Expt. 3 are given as means (circles) with twice the standard deviation (vertical lines).

From the graphs in Figure 3 it can be seen that there is a rapid decline in the number of flower primordia formed with the intercalation of a single long day in between single short days, or between pairs of short days. Indeed, the fall is so great that there is, as Long (1939) found, little or no summation with repetitions of single short and single long days. Further, the level reached in groups J. K and L (where 5 groups each of two short days were given) is almost exactly five times that reached in group C, which had only two short days. This again corroborates Long's findings — although in the more juvenile plants with which he experimented it was not possible to demonstrate it for paired short days. On the other hand, the levels reached with 6 or with 10 consecutive short days are much higher than 3 or 5 times that with 2 short days. However, the rate of ascent of flower number with increasing numbers of consecutive short days, which Hamner (1940) found to be linear up to seven short days, begins to fall off above that number, so that the difference between 6 and 10 short days is not as great as that between 2 and 6 short days. It may be tentatively suggested that this is due to the limitation of the number of »sites» at which flower primordia can be formed. Borthwick and Parker (1938) have shown that only terminal apices which have not yet formed leaf primordia are converted to flowering apices during induction of *Biloxi soybean*.

Discussion

These experiments serve to amplify the conclusions which follow from an examination of previous work on summation of photoperiodic stimuli, namely that summation is possible in both long-day and short-day plants.

Two distinct processes may be envisaged as determining whether or not summation does take place in any given plant. The first process concerns the immediate fate of the substances produced by the leaf exposed to the inductive photoperiodic cycle. These substances may be relatively unstable in parts of the plant other than shoot apical meristems (Carr 1953; I). Alternatively, these products may be neutralised by some system operating in non-inductive cycles, so that only those amounts of the precursors of the flower hormone which reach the apical meristem are permanently retained. In Biloxi soybean the destructive or antagonistic mechanism is so potent that a single short day has no permanent effect, but in *Chenopodium amaranticolor* summation is quite effective over at least 4 long days. The same is true of *Xanthium* (Long 1939).

The second process limiting summation concerns the diluting effect of continued cell division in the apical meristem. On Gregory's (1948) view (see Carr 1953; I) only when a *critical* level of the precursor of flowering has accumulated in the apical meristem is further production autocatalytic. Under normal conditions, the attainment of this level would appear to necessitate only a single short-day cycle in *Xanthium pennsylvanicum*, but three long-day cycles in *Hyoscyamus niger*. Assuming that the critical concentration for autocatalytic production of flower hormone has not been attained, then continued cell division over long periods must result in the depletion of the store of flower hormone to a completely ineffective concentration. Thus, in *Hyoscyamus niger* the »sub-threshold« effect of a pair of long days is lost over a period of 65 short days, or at least does not result in autocatalytic production to such a level that another long day would suffice to turn the balance towards flowering. Summation is the less effective the smaller are the residues of individual photoperiodic stimuli remaining after long intervening periods of non-inductive conditions. We have to distinguish, then, between the dissipating effects of continued cell division in the apical meristems and the relatively short-term effects of some system which, operating optimally in optimally non-inductive conditions, antagonises or actively breaks down the flowering hormone or its precursors.

It is possible that the well-known increase in photoperiodic sensitivity with age may represent an ontogenetic decline in this antagonistic system, and such an explanation may well be capable of extension to the responses of faculta-

tively long-day and short-day plants. There is a great need for further information on the effects of age, light intensity and temperature on the decay of the photoperiodic stimulus.

Summary

Previous work on summation of photoperiodic stimuli is reviewed, and it is shown that summation is possible in both short-day and long-day plants. This is supported by experiments on *Hyoscyamus niger*, a long-day plant, and *Chenopodium amaranticolor*, a short-day plant. Long's findings on summation in Biloxi soybean are confirmed and amplified.

The experiments show that the product of an inductive photoperiodic stimulus may decay at a rate which is determined largely by the plant species. Evidence from earlier work shows that light and darkness affect the rate of decay, but probably temperature is also involved. In accordance with Gregory's view it is suggested that autocatalytic production of flower hormone does not begin unless a critical level of precursor has accumulated in the apical meristems, and this level can be reached by summing sub-critical concentrations. Summation is the less effective the smaller are the residues of individual photoperiodic stimuli remaining after long periods of non-inductive conditions. Within the apical meristems the precursor may be protected from decay, but over *long* periods of time sub-critical amounts may be dissipated by cell division and repartition. In both short-day and long-day plants the residue of a sub-threshold photoperiodic treatment may thus be virtually lost.

Decay of the flowering stimulus over *short* periods is attributed to some destructive or antagonistic system within the plant, and changes in this system are tentatively held responsible for certain ontogenetic changes in plant responses to daylength.

References

- Borthwick, H. A. & Parker, M. W.: Influence of photoperiods upon the differentiation of meristems and the blossoming in Biloxi soybean. — *Bot. Gaz.* 99: 825. 1938.
Carr, D. J.: On the Nature of Photoperiodic Induction, I & II. *Physiol. Plant.* 6: 672, 680. 1953.
Claes, H. & Lang, A.: Die Blütenbildung von *Hyoscyamus niger* in 48-stündiger Licht-Dunkel-Rhythmen und in Zyklen mit aufgeteilter Lichtphasen. — *Zeit. f. Naturf.* 2 b: 56. 1947.
Garner, W. W. & Allard, H. A.: Effect of abnormally long and short alternation of light and darkness on growth and development of plants. — *J. Agr. Res.* 18: 42, 629. 1931.
Gregory, F. G.: The control of flowering in plants. — *Symp. Soc. Exp. Biol. Cambr.* II. Growth. 75. 1948.

- Hamner, K. C.: Interrelation of light and darkness in photoperiodic induction. — *Bot. Gaz.* 101: 658. 1940.
- Harder, R. & Gümmer, G.: Über die Blütenbildung von *Kalanchoë Blossfeldiana* in verschiedene Licht-Dunkel-Rhythmen. — *Planta* 37: 12. 1949.
- Lang, A.: Entwicklungsphysiologie. — *Fortschritte der Botanik* 12: 340. 1949.
- Physiology of flowering. — *Ann. Rev. Plant. Physiol.* 3: 340. 1952.
- & Melchers, G.: Die photoperiodische Reaktion von *Hyoscyamus niger*. — *Planta* 33: 653. 1943.
- Lona, F.: Correlazione di sviluppo e di accrescimento fra parti di una stessa pianta (di tipo longidiurna) tenute in condizione fotoperiodica differenti. — *Lavori di Botanica* (G. Gola Jubilee Vol.) Padua: 285. 1947 b.
- Il significato dei glucidi e del fattore antiflorigeno nel meccanismo di fioritura delle pianta erbacee ed in particolare delle brevidiurne. — *Rend. Inst. Lombardo di Sci. e Lett.* III. 83: 1. 1950 c.
- Long, E. M.: Photoperiodic induction as influenced by environmental factors. — *Bot. Gaz.* 101: 168. 1939.
- Melchers, G. & Lang, A.: Die Physiologie der Blütenbildung. — *Biol. Zbl.* 67: 105. 1948.
- Naylor, A. W.: Effects of some environmental factors on photoperiodic induction of beet and dill. — *Bot. Gaz.* 102: 557. 1941.
- Post, K.: Accumulation of photoperiodic stimuli in *Chrysanthemums*. — *Proc. Am. Soc. Hort. Sci.* 55: 425. 1950.
- Potapenko, A.: (The origin of the photoperiodic reaction and its significance in regard to adaptation). — *Compt. Rend. (Doklady) Acad. Sci. URSS.* 57: 959. 1947.
- Roberts, R. H.: Induction and blossoming of *Xanthium*. — *Science* 113: 726. 1951.
- Rupcheva, I. A.: (Development of plants under conditions of alternating photoperiodic cycles). — *Compt. Rend. (Doklady) Acad. Sci. URSS.* 61: 74. 1948.
- Samygin, G. A.: (The effect of light intensity in photoperiods unfavourable to the development of *Rudbeckia* and *Perilla*). — *Compt. Rend. (Doklady). Acad. Sci. URSS.* 60: 1433. 1948.

Der Einfluss des Heteroauxins auf die Plasmapermeabilität für Harnstoff und Alkylharnstoffe

Von

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I. Einleitung

Der Mechanismus der Auxin-Wirkung in Pflanzen, besonders beim Metabolismus, d.h. Atmung usw., ist viel bearbeitet worden. Die Auxin-Wirkung auf den Kolloidzustand des Protoplasmas ist jedoch fast noch nicht erforscht. Vor kurzen ist gezeigt worden, dass das Auxin auf die Permeabilität des Protoplasmas einen Einfluss ausübt (Guttenberg *et al.*, 1951, 1952 a, b; Masuda, 1953, 1955).

Andererseits sind die Fragen, die die Permeabilität betreffen, ebenfalls bearbeitet worden. Collander und Wikström (1949 a, b) haben gezeigt, dass die Protoplasamembran in gewissem Sinne einen Siebeffekt für die kleinsten Moleküle zeigt und andererseits haben sie durch Messung der Permeabilität pflanzlicher Protoplasten für Harnstoff und Alkylharnstoffe bewiesen, dass die Protoplasamembran nicht nur Porenpermeabilität sondern auch Lipidpermeabilität zeigt.

Bezüglich des Einflusses von Auxin auf die Permeabilität haben Guttenberg *et al.* (1951, 1952 a, b) gezeigt, dass Auxin die Permeabilität der Epidermiszellen der Blattunterseite von *Rhoeo discolor* für Wasser und Zucker beeinflusst und dadurch beweisen, dass das Auxin auf die Eiweissphase der Protoplasamembran, d.h. die Porenpermeabilität eine Wirkung hat. Dagegen hat Masuda (1953, 1955) bewiesen, dass das Auxin die Permeabilität für Harnstoff und Glyzerin der Innenepidermiszellen der Zwiebelschuppen von *Allium cepa* und der Koleoptilen von *Avena sativa* beeinflusst, und deshalb

vermutet, dass das Auxin wahrscheinlich nicht nur auf die Eiweissphase sondern auch auf die Lipoidphase der Protoplasmamembran wirkt. Um diese Vermutung zu bestätigen ist es jedoch erforderlich, dass man den Einfluss des Auxins auf die Permeation für die homologe Reihe der gelösten Stoffe, die verschiedene Lipoidlöslichkeiten haben, bestimmt.

In der vorliegenden Arbeit wählte ich als permeierende Stoffe die homologe Reihe der Alkylharnstoffe, d.h. Harnstoff (HS), Methylharnstoff (MH) und Äthylharnstoff (AH).

II. Material und Methode

Als Untersuchungsobjekt wurden die Innenepidermiszellen der Zwiebelshuppen von *Allium cepa* und die des wachsenden Teiles der etiolierten Koleoptilen von *Avena sativa* gewählt.

Im Falle von *Allium* wurden die herausgeschnittenen Zwiebelshuppen von etwa 10 mm Kantenlänge in bidest. Wasser bzw. Heteroauxinlösung überführt und darin 1—2 Stunden liegen gelassen. Dann wurden die Oberflächenepidermisplättchen von etwa 5 mm Kantenlänge von dem Zwiebelshuppenwürfel herausgeschnitten. Diese Schnitte wurden ins Plasmolytikum gebracht, das mit oder ohne Zufügung gleicher Konzentration des Heteroauxins hergestellt war. Im Falle von *Avena*, wie in Abb. 1 a gezeigt wird, wurde je ein 4 mm langes Zylinderstück, das von der Spitze der etiolierten 20—30 mm langen Koleoptile 4—5 mm entfernt war, abgeschnitten. Es wurde in bidest. Wasser oder in die Heteroauxinlösung, die 3 % Saccharose enthielt, überführt und darin 3—4 Stunden gewässert (Masuda, 1955). Dann wurden von diesen Koleoptilzylindern die Schnitte, die, wie Abb. 1 b zeigt, die Leitbündel nicht enthalten, herausgeschnitten und ins Diosmotikum gebracht.

Für die Permeabilitätsbestimmungen verwendete ich die Permeationskonstante P' nach der Deplasmolysezeit-Methode von Hofmeister (Pd', 1948) und die plasmometrische Methode von Url (1951, 1952), die auf den früheren Angaben von Höfler (1918) beruht. Die erstere Methode wurde bei *Allium* und die letztere bei *Avena* verwendet.

Die Pd' von Hofmeister ist die Permeationskonstante P' , die aus der Deplasmolysezeit gewonnen wird und ergibt sich im Folgenden nur aus Formel

$$\frac{120 (C-O)}{(C+O)}, \text{ wo } P' = \frac{M}{C-c}, M = \frac{(C-O) 60}{T}, c = \frac{C-O}{2},$$

C = Konzentration des Plasmolytikums, c = jeweilige Konzentration der eingedrungenen Substanz im Zellsaft, M = die pro Stunde aufgenommene Menge der permeierenden Substanz in Mol., O = osmotischer Wert des Objektes, T = Deplasmolysezeit ist. Der O -Wert wurde in folgender Weise gemessen: die gleichartigen Epidermisstücke wurden in Glukoselösungen mit verschiedenen Konzentrationen gebracht und dadurch der O -Wert in der Konzentration bestimmt, in der 50 % der Zellen plasmolysiert waren. Die Deplasmolysezeit ist die Zeit, die mehr als 95 % Zellen brauchen um zu deplasmolysieren.

Bei *Avena*, nach Url (1951, 1952), diente als Mass der Permeabilität die Grösse ΔG , welches die Änderung des Plasmolysegrades pro Stunde angibt und die Differenz von zwei Plasmolysegraden G_1 und G_2 ist, welche mit einem Zeitabstand von einer

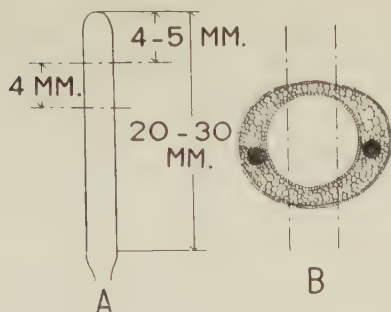


Abb. 1.

Stunde gemessen werden. Dabei ist $G_2 - G_1 = \frac{l_2 - l_1}{h}$, da der G-Wert $\frac{l-b/3}{h}$ ist, wenn l = die Länge des Protoplasten, h = die innere Länge der Zelle, und b = die innere Breite der Zelle bedeutet.

Um die schädigenden Wirkungen der Diosmotika zu vermeiden wurde in allen Fällen der »Partialversuch« von Hofmeister (1935) verwendet und mit dem »Totalversuch« verglichen. Um die von den Diosmotika verursachten abnormen Permeabilitätssteigerung zu vermeiden, wurde weiterhin noch die *gleichzeitige* Methode von Collander und Wikström (1949 a, b) verwendet, in der man Harnstoff und Methylharnstoff *gleichzeitig* durch *dieselben* Protoplasten diffundieren lässt.

Die Konzentrationen der Indolessigsäure (im Folgenden mit IES abgekürzt), die in diesen Versuchen verwendet wurden, waren bei *Allium* 0.01 mg/l, welches die Permeabilität vergrößert, und 100 mg/l, welches sie hemmt; und bei *Avena* 1 mg/l, was die Permeabilität sowie die Zellstreckung fördert (Masuda, 1953, 1955).

Die verwendeten IES, IIS, MH und AH waren reinste Präparate von Merck, Schuchardt, Wako bzw. Katayama. Sie wurden in bidest. Wasser gelöst.

III. Versuchsergebnisse

1) *Allium cepa*

a) Totalversuch

Die Hauptversuchsreihe mit dieser Pflanze führte ich zwischen 2. und dem 20. September 1954 durch.

Ein Beispiel der durch den Totalversuch gewonnenen Pd'-Messungsergebnisse der Innenepidermiszellen wird im Folgenden gegeben:

Harnstoff

3. September 1954

C (Konzentration des Diosmotikums) = 0.55 Mol. Harnstoff, $t = 28^\circ \text{C}$.

Schnitte	Osmotischer Wert (O)	Deplasmolysezeit (T)	$\frac{(C-O)60}{T}$	$\frac{C-O}{2}$	Pd'
1	0.45 Mol.	85 Min.	0.0706	0.050	0.141
2	0.42	100	0.0780	0.065	0.161
3	0.43	85	0.0847	0.060	0.141

Mittel = 0.148

Tabelle 1. *Pd'-Werte in den Totalversuchen der Innenepidermiszellen von Allium cepa.*

	Harnstoff		Methylharnstoff		Aethylharnstoff	
	Pd'	IES/K	Pd'	IES/K	Pd'	IES/K
Kontrolle	0.15	—	0.63	—	1.89	—
0.01 mg/l IES	0.19	1.27	0.88	1.40	2.90	1.53
100 mg/l IES	0.11	0.73	0.43	0.68	0.96	0.51

Die Tabelle 1 zeigt die Mittelwerte aus allen Versuchen der Hauptreihe.

Sie zeigt, dass die Permeabilitätsreihe $AH > MH > HS$ ist. Es ist dabei bemerkenswert, dass alle diese Stoffe in die Zelle, der 0.01 mg/l IES gegeben wird, schneller und in die Zelle, der 100 mg/l IES gegeben wird, langsamer eindringen als die Kontrolle. Die Tabelle zeigt weiterhin, dass durch 0.01 mg/l IES die Permeabilität für MH mehr als die für HS und die für AH mehr als die für MH erhöht wird, und sie zeigt auch, dass durch 100 mg/l IES die Permeabilität für diese Stoffe der Reihe nach erniedrigt wird.

In diesem Falle ist es zu befürchten, dass der MH manchmal eine deutliche Tendenz hat, die Durchlässigkeit der Protoplasmamembran zu erhöhen (Pecksieder, 1947; Collander und Wikström, 1949 a). Deshalb wurde der Partialversuch ausgeführt.

b) Partialversuch

Die Hauptversuchsreihe führte ich zwischen dem 22. September und dem 6. Oktober 1954 durch.

Die Lösungen enthielten 0.2 Mol. Glukose – 0.35 Mol. HS oder 0.8 Mol. MH bzw. AH (Tabelle 2).

Da sich die Pd' -Werte für MH und AH in den Partialversuchen kleiner als die in den Totalversuchen ergeben, muss man beachten, dass eine sekundäre Erhöhung der Protoplasmapermeabilität, die während der Permeation dieser Stoffe eintreten kann, existiert. Es ist aber bemerkenswert, dass die Quotienten IES/K für HS, MH und AH in Tabelle 2 im Prinzip denen des Totalversuches ganz ähnlich sind.

Ich verwendete auch die *gleichzeitige* Methode, um festzustellen, ob die

Tabelle 2. *Pd'-Werte in den Partialversuchen der Innenepidermiszellen von Allium cepa.*

	HS		MH		AH	
	Pd'	IES/K	Pd'	IES/K	Pd'	IES/K
Kontrolle	0.16	—	0.43	—	0.96	—
0.01 mg/l IES	0.19	1.13	0.60	1.40	1.50	1.56

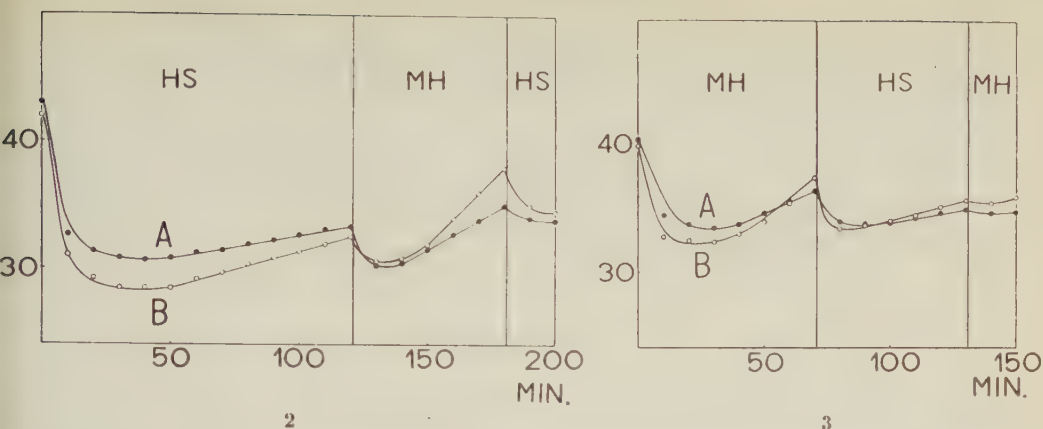


Abb. 2 und 3. Plasmolyseverläufe der Innenepidermiszellen von *Allium cepa* in der Harnstoff-(HS) und Methylharnstofflösung (MH). A: Kontrolle, B: 0.01 mg/l IES. Abszisse: Zeit in Minuten. Ordinate: Protoplastenlänge in Mikrometerskalenteilen.

Permeabilitätseigenschaften der Protoplasten durch MH oder AH abnorm werden oder nicht.

Das Innenepidermisplättchen wurde nach vorangehender Wässerung in eine Durchflussskammer gebracht. Hier wurde es zunächst in einer Lösung plasmolysiert, die in einem Liter Lösung 0.2 Mol. Glukose + 0.8 Mol. HS enthielt (Lösung I). Die Protoplasten rundeten sich in kurzer Zeit ab und fingen an, sich allmählich auszudehnen. Die Inklinationen der Abstiegskurven in Abb. 2 zeigen die Austrittsgeschwindigkeit des Wassers und die der Aufstiegskurven hauptsächlich die Eintrittsgeschwindigkeit des HS. Das Präparat wurde nun in die Lösung II gebracht, deren Zusammensetzung genau dieselbe war wie die erste ausser dass HS durch MH ersetzt war. In diesem Falle waren die Ausdehnungsgeschwindigkeiten grösser als die Lösung I. Ersetzte man die Lösung II wieder durch die Lösung I, so zeigte es sich, dass der Filtrierwiderstand der Plasmamembran nicht bedeutend gestört worden war. Wenn das Material aber zunächst in der Lösung II plasmolysiert und dann in die Lösung I gebracht wurde (Abb. 3), zeigten die Aufstiegskurven keine steilen Inklinationen. Wenn die Plasmamembran in dem MH 70 Min. lang plasmolysiert wurde und sich ziemlich ausgedehnt hatte, konnte man beobachten, dass keine merkliche Störung der HS-Permeabilität eintritt. In den Totalversuchen ist es vielleicht zu befürchten, dass MH oder AH eine sekundäre Permeabilitätserhöhung verursacht, aber in den Partialversuchen braucht man eine solche eventuelle Möglichkeit nicht in Betracht zu ziehen.

Wenn 0.01 mg/l IES in die Lösung I und II gegeben wurde, wie Abb. 2 B und 3 B zeigen, waren die Wasseraustritts- und Stoffeintrittsgeschwindig-

keiten grösser als die der Kontrolle, und zwar war dieser Effekt von IES auf die Permeabilität für MH grösser als auf die Permeabilität für HS. Die Ergebnisse in den Total- und Partialversuchen wurden auch hier sicher gestellt.

Da in einer Lösung, die den AH enthielt, die Ausdehnungsgeschwindigkeit der Protoplasten zu gross war, konnte ich die Versuche, bei denen AH verwendet wurde, nicht messend verfolgen.

2) *Avena sativa*

Die Hauptversuchsreihe mit dieser Pflanze führte ich zwischen dem 3. November und dem 29. Dezember 1954 durch.

Die Messungen der einzelnen Zellen erfolgten in genauen Zeitabständen; jede Minute, mitunter alle 30 Sekunden, wurde die Protoplastenlänge einer Zelle gemessen (Url, 1951, 1953). Als Beleg teile ich folgendes Streng vergleichbares Versuchspaar mit.

a) Totalversuch

Harnstoff

20. November 1954

C (Konzentration des Osmotikums) = 1 Mol. HS, $t = 18^\circ \text{C}$.

Das Koleoptilstückchen wurde nach einer Wässerung von 3 Stunden um 1^h 15' in die Lösung gebracht (Kontrolle), und ein anderes wurde nach der in der Auxin-Lösung geführten Wässerung von 4—5 Stunden um 3^h 05' in die Lösung gebracht, die auch das gleiche Auxin enthielt.

Kontrolle, 1. Messung 1^h 36', 2. Messung 1^h 46', 3. Messung 1^h 56'.

Zelle	h	b	l ₁	l ₂	l ₃	G ₁	G ₂	G ₃	ΔG_{2-1}	ΔG_{3-2}
1	37	8.1	24	25	26	0.5757	0.6027	0.6297	0.162	0.162
2	44	8.4	27.4	28.6	30	0.5591	0.5864	0.6182	0.164	0.191

ΔG (Mittel) = 0.170

1 mg/l IES, 1. Messung 3^h 25', 2. Messung 3^h 30', 3. Messung 3^h 35'.

Zelle	h	b	l ₁	l ₂	l ₃	G ₁	G ₂	G ₃	ΔG_{2-1}	ΔG_{3-2}
1	58	8.4	33	33.9	34.9	0.5207	0.5362	0.5535	0.186	0.208
2	74	8.4	40	41.2	42.3	0.5027	0.5189	0.5338	0.195	0.191

ΔG (Mittel) = 0.195

Methylharnstoff

16. November 1954

Die Schnitte wurden nach Wässerungen um 3^h 55' (Kontrolle) und 2^h 45' (IES) in die Lösungen gebracht, C = 1 Mol. Methylharnstoff, $t = 16^\circ \text{C}$.

Kontrolle. 1. Messung 4h 17', 2. Messung 4h 19', 3. Messung 4h 21'.

Zelle	h	b	l ₁	l ₂	l ₃	G ₁	G ₂	G ₃	ΔG_{2-1}	ΔG_{3-2}
1	38	9	34.5	35.8	37.1	0.8289	0.8632	0.8974	1.029	1.026
2	46	8.1	40.8	42.2	43.7	0.8283	0.8587	0.8913	0.912	0.978

 ΔG (Mittel) = 0.986

1 mg/l IES 1. Messung 2h 56', 2. Messung 2h 58', 3. Messung 3h.

Zelle	h	b	l ₁	l ₂	l ₃	G ₁	G ₂	G ₃	ΔG_{2-1}	ΔG_{3-2}
1	53	9.9	42.6	44.3	46.5	0.7415	0.7736	0.8151	0.963	1.245
2	41	6.9	32.8	34.4	36	0.7439	0.7829	0.8219	1.170	1.170

 ΔG (Mittel) = 1.137

Tabelle 3 gibt die Mittelwerte aus allen Versuchen der Hauptreihe. Aus diesen Ergebnissen kann man erkennen, dass der MH im Durchschnitt in die Zellen etwa 6 mal schneller eindringt als der HS. Weiterhin ist die Permeabilität für beide Stoffe durch IES etwa 1.3 mal grösser als die in der Kontrolle gefundene Permeabilität.

Tabelle 3. ΔG -Werte in den Totalversuchen von *Avena Koleoptilen*.

	HS		MH	
	ΔG	IES/K	ΔG	IES/K
Kontrolle	0.165	—	0.981	—
1 mg/l IES	0.207	1.25	1.247	1.27

Da in diesem Falle auch die Gefahr einer sekundären Erhöhung der Protoplasmapermeabilität durch die konzentrierten Diosmotika bestand, wurde wieder die Partialversuch-Methode verwendet.

b) *Partialversuch*

Harnstoff

17. Dezember 1954

Die Schnitte wurden nach Wässerungen um 2h 30' (Kontrolle) und 4h 30' (IES) in die Lösungen gebracht. C=0.2 Mol. Glukose + 0.8 Mol. Harnstoff. t=22° C.

Kontrolle. 1. Messung 2h 52', 2. Messung 3h 02', 3. Messung 3h 12'.

Zelle	h	b	l ₁	l ₂	l ₃	G ₁	G ₂	G ₃	ΔG_{2-1}	ΔG_{3-2}
1	45	8.1	28.5	30	31.5	0.5733	0.6067	0.6400	0.200	0.200
2	42	7.5	26.7	28	29.4	0.5762	0.6071	0.6405	0.185	0.200

 ΔG (Mittel) = 0.196

1 mg/l IES 1. Messung 4h 48', 2. Messung 4h 53', 3. Messung 4h 58'.

Zelle	h	b	l ₁	l ₂	l ₃	G ₁	G ₂	G ₃	ΔG_{2-1}	ΔG_{3-2}
1	34	8.1	28.2	28.9	29.5	0.7500	0.7706	0.7882	0.247	0.211
2	48	8.1	39	39.9	40.6	0.7562	0.7730	0.7900	0.203	0.204

 ΔG (Mittel) = 0.216

Methylharnstoff

18. Dezember 1954

Die Schnitte wurden nach Wässerungen um 1^h 30' (Kontrolle) und 2^h 13' (IES) in die Lösungen gebracht. C=0.2 Mol. Glukose+0.8 Mol. Methylharnstoff. t=22° C.

Kontrolle. 1. Messung 1^h 46', 2. Messung 1^h 48', 3. Messung 1^h 50'.

Zelle	h	b	l ₁	l ₂	l ₃	G ₁	G ₂	G ₃	ΔG_{2-1}	ΔG_{3-2}
1	52	9.6	46	47.5	49.1	0.8231	0.8519	0.8827	0.864	0.924
2	34	6.9	30.2	31.1	32.1	0.8206	0.8471	0.8765	0.795	0.882

ΔG (Mittel) = 0.866

1 mg/l IES 1. Messung 2^h 27', 2. Messung 2^h 29', 3. Messung 2^h 31'.

Zelle	h	b	l ₁	l ₂	l ₃	G ₁	G ₂	G ₃	ΔG_{2-1}	ΔG_{3-2}
1	82	9	64	66.9	69.8	0.7439	0.7793	0.8146	1.062	1.059
2	33	7.8	27.3	28.5	29.5	0.7485	0.7848	0.8152	1.089	0.912

ΔG (Mittel) = 1.055

Da AH in die Zellen zu schnell eindrang, ohne die vollständige konvexe Plasmolyse zu zeigen, d.h. die Ausdehnungsgeschwindigkeit der Protoplasten zu gross war, konnte ich keine genauen Werte von ΔG erhalten.

Tabelle 4 gibt die Mittelwerte aus allen Versuchen der Hauptreihe. Aus diesen Ergebnissen kann man eine ganz ähnliche Tendenz wie bei den Totalversuchen erkennen, nur dass das Verhältnis des ΔG -Wertes von MH zu dem von HS hier kleiner ist als in den Totalversuchen.

Tabelle 4. ΔG -Werte in den Partialversuchen von *Avena Koleoptilen*.

	HS		MH	
	ΔG	IES/K	ΔG	IES/K
Kontrolle	0.190	—	0.876	—
1 mg/l IES	0.229	1.21	1.081	1.23

Diese Ergebnisse von *Avena* unterscheiden sich von den mit *Allium* gefundenen, da fast kein Unterschied zwischen der durch IES entstandenen Permeabilitätssteigerung für MH und derjenigen für HS in den Total- und Partialversuchen gefunden wurde.

IV. Besprechung der Ergebnisse

Der Einfluss des Auxins auf die Permeabilität ist darauf zurückzuführen, dass das Auxin unmittelbar oder mittelbar den Kolloidzustand der Protoplasamembran verändert.

Es ist bekannt, dass es Pflanzen gibt, wie z.B. *Beggiatoa*, bei denen die Porenpermeabilität besonders hervortritt (Schönfelder, 1930), aber dass die meisten Hochpflanzen ausserdem die Lipoidpermeabilität zeigen (Collander, 1949 b). In welchem Grade die Molekülgrösse und die Lipoidlöslichkeit permeierender Stoffe ausschlaggebend sind wird wohl von den Materialien und ihren inneren oder äusseren Bedingungen abhängig sein (Collander, 1937). Von diesen beiden Ausgangspunkten will ich nun meine Ergebnisse besprechen.

Die Molekülgrösse (MR_D) und die Verteilungen zwischen Oel und Wasser des HS, MH und AH sind nach Ruhland und Hoffmann (1925) und Collander und Bärlund (1933), wie folgt:

	MR_D	Aether/Wasser	Olivenöl/Wasser	$\frac{\text{Olivenöl} + \text{Oelsäure}}{\text{Wasser}}$
HS	13.67	0.0005	0.00015	0.0052
MH	18.47	0.0012	0.00044	0.0096
AH	$22 \ll 30$	0.0041	0.0017	0.034

Je mehr die Molekülgrösse und die Lipoidlöslichkeit in dieser homologen Reihe zunehmen, desto mehr nimmt auch die Permeation der Zellen von *Allium* und *Avena* zu. Deshalb kann man sagen, dass die Lipoidlöslichkeit in diesen Materialien und die Versuchsbedingungen einen wesentlichen Einfluss haben.

Da in den Totalversuchen der Einfluss der permeierenden Substanzen auf die Permeabilität erkannt wurde, so ist der Vergleich der relativen Werte der Permeabilität zwischen der homologen Reihe den Partialversuchen mit den Werten der Verteilungskoeffizienten zwischen Oel und Wasser wie folgt:

	Verteilungskoeffizient			<i>Allium</i>		<i>Avena</i>	
	$\frac{\text{Aether}}{\text{Wasser}}$	$\frac{\text{Oel}}{\text{Wasser}}$	$\frac{\text{Oel} + \text{Oelsäure}}{\text{Wasser}}$	Kont.	IES	Kont.	IES
MH/HS	2.4	2.9	1.9	2.7	3.2	4.6	4.7
AH/MH	3.4	3.9	3.5	2.2	2.5	—	—

Falls IES nicht beigegeben wurde, waren die Permeationen von MH und HS in den Innenepidermiszellen von *Allium* hauptsächlich von der Lipoidlöslichkeit abhängig, aber die Molekülgrösse scheint auf die AH-Permeation einzuwirken.

Bei Anwesenheit von IES wurden die Quotienten der Permeabilität grösser. Das deutet an, dass IES die Lipoidphase der Protoplasmamembran starker beeinflusst als die Wasserphase, die eine Rolle als Ultrafilter spielt.

Es ist bedeutungsvoll, dass der permeabilitätserhöhende Effekt der IES in der optimalen Konzentration die Ordnung $AH > MH > HS$ hat. Der permeabilitätserniedrigende Effekt der IES in der hohen Konzentration zeigt dieselbe Ordnungsfolge. Die gleichzeitige Methode zeigt aber, dass IES nicht nur die Lipoidpermeabilität, sondern auch die Wasserdurchlässigkeit erhöht.

Bei Avena Koleoptile, auch wenn IES nicht beigegeben wurde, ist der Quotient MH/HS bedeutend grösser, daher hat die Steigerung der Lipoidlöslichkeit auf die Permeation einen grösseren Effekt. Avena unterscheidet sich von Allium dadurch, dass der permeabilitätserhöhende Effekt von IES keinen deutlichen Unterschied zwischen HS und MH zeigt. Man kann sich die Ursache hierfür in verschiedener Weise denken, z.B. 1) der Unterschied der verwendeten Konzentrationen von IES, 2) bei Avena wurden im Gegensatz zu Allium die lebhaft wachsende Zellen verwendet, bei Allium ist es auch möglich, dass es einen durch die Jahreszeit bedingten Unterschied gibt, 3) da bei Avena schon bei der Kontrolle die maximale Lipoidpermeabilität auftritt, kann die weitere Erhöhung der Permeabilität durch IES schwer hervortreten, 4) der Unterschied zwischen den Materialien. Zur Entscheidung, welche von diesen Vorschlägen der ausschlaggebende ist, sind weitere Untersuchungen nötig.

Es wäre interessant zu wissen, in welcher Weise die Permeabilitätsänderungen durch IES entstehen, und welche von diesen beiden, nämlich die durch IES entstandenen Permeabilitätsänderungen oder die durch IES hervorgerufenen Kolloidzustandsänderungen, die Zellaktivität erhöhen oder *vice versa*.

V. Zusammenfassung

1. Die Permeabilitätsreihen der Innenepidermiszellen der Zwiebeln von *Allium cepa* und die der Koleoptilen von *Avena sativa* sind wie folgt: Aethylharnstoff (AH) > Methylharnstoff (MH) > Harnstoff (HS).

2. Bei Allium wurde die Permeabilität für diese Stoffe durch 0.01 mg/l bzw. 100 mg/l IES erhöht bzw. erniedrigt. In allen Fällen dieser beiden IES-Effekte sind ihre Stärke wie folgt: $AH > MH > HS$. IES erhöht nicht nur die Wasserpermeabilität, sondern sie verändert auch stark die Lipoidphase der Protoplasmamembran.

3. Bei Avena erhöht 1 mg/l IES die Permeabilität, der Effekt zeigt aber keinen Unterschied zwischen MH und HS.

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Hier sei es mir gestattet, meinem verehrten Lehrer, Herrn Prof. Joji Ashida, Kyoto Universität, für seine Anregung und ständige Anleitung den besten Dank auszusprechen.

Literatur

- Collander, R. & Bärlund, H.: Permeabilitätsstudien an *Chara ceratophylla*. — Acta Bot. Fenn. 11: 1. 1933.
- The Permeability of Plant Protoplasts to Non-Electrolytes. — Trans. Faraday Soc. 33: 985. 1937.
- & Wikström, B.: Die Permeabilität pflanzlicher Protoplasten für Harnstoff und Alkylharnstoffe. — Physiol. Plantar. 2: 235. 1949 a.
- The Permeability of Plant Protoplasts to Small Molecules. — Ibid. 2: 300. 1949 b.
- Guttenberg, H. v. & Beythien, A.: Ueber den Einfluss von Wirkstoffen auf die Wasserpermeabilität des Protoplasmas. — Planta 40: 36. 1951.
- und Meinl, G.: Ueber den Einfluss von Wirkstoffen auf die Wasserpermeabilität des Protoplasmas. II. Mitteilung. — Ibid. 40: 431. 1952 a.
- — Ueber den Einfluss von Wirkstoffen auf die Wasserpermeabilität des Protoplasmas. III und IV. Mitteilung. — Ibid. 41: 167. 1952 b.
- Höfler, K.: Permeabilitätsbestimmung nach der plasmometrischen Methode. — Ber. D. Bot. Ges. 36: 414. 1918.
- Hofmeister, L.: Vergleichende Untersuchungen über spezifische Permeabilitätsreihen. — Bibliothica Bot. 113: 1. 1935.
- Ueber Permeabilitätsbestimmung nach der Deplasmolysezeit. — Sitzungsber. Oesterr. Akad. Wiss. Wien, Math.-nat. Klasse, Abt. I, 157: 83. 1948.
- Masuda, Y.: Ueber den Einfluss von Auxin auf die Stoffpermeabilität des Protoplasmas. I. Mitteilung. — Bot. Mag. Tokyo, 66: 256. 1953.
- Ueber den Einfluss von Auxin auf die Stoffpermeabilität des Protoplasmas. II. Mitteilung. — Ibid. 1955. (im Druck).
- Pecksieder, M. E.: Permeabilitätsstudien an Lebermoosen. — Sitzungsber. Oesterr. Akad. Wiss. Wien, Math.-nat. Klasse, Abt. I, 156: 521. 1947.
- Ruhland, W. & Hoffmann, C.: Die Permeabilität von *Beggiatoa mirabilis*. — Planta 1: 1. 1925.
- Schönfelder, S.: Weitere Untersuchungen über die Permeabilität von *Beggiatoa mirabilis* usw. — Planta 12: 414. 1930.
- Url, W.: Permeabilitätsverteilung in den Zellen des Stengels von *Taraxacum officinale* und anderer krautiger Pflanzen. — Protopl. 40: 475. 1951.
- Unterschiede der Plasmapermeabilität in den Gewebeschichten krautiger Stengel. — Physiol. Plant. 5: 135. 1952.

Molybdenum in Relation to Nitrogen Metabolism

I. Assimilation of Nitrate Nitrogen by *Scenedesmus*

By

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Introduction

Although the status of molybdenum as an essential micronutrient for higher plants is well established, its role in the nutrition of green algae is less well documented. Recent reviews of mineral requirements of green algae could adduce no evidence for the essentiality of molybdenum (Myers, 19; Ketchum, 15). More recently, however, evidence was presented for a molybdenum requirement of *Chlorella pyrenoidosa* (Walker, 26; Loneragan and Arnon, 16) and *Scenedesmus obliquus* (Arnon *et al.*, 6). This paper reports further studies on molybdenum in the nutrition of green algae with special reference to the nitrogen metabolism of *Scenedesmus obliquus* (Strain D3).

The conclusive identification of molybdenum as the specific metal component of nitrate reductase from *Neurospora crassa* by Nicholas and Nason (20, 21) has rendered it probable that this metal is also an essential constituent of nitrate reductase in green plants. This would explain the widely observed accumulation of nitrate in molybdenum-deficient plants and its disappearance upon the addition of molybdenum (Mulder, 18; Hewitt and Jones, 11; Meagher, 17; Spencer and Wood, 23).

If molybdenum were needed by a green plant solely for nitrate reduction, then the requirement for this micronutrient should be abolished with the substitution of a reduced form of nitrogen for nitrate. A hitherto unobserved clear-cut dependence of only one enzyme system on a specific inorganic

element, essential for growth, would be established. A micronutrient would thus be made wholly dispensable through bypassing the specific »metabolic block» caused by its deficiency. Molybdenum seems to fit this pattern in the nutrition of *Scenedesmus*. This paper presents evidence for the essentiality of molybdenum with nitrate as the sole source of nitrogen. The companion communication (Ichioka and Arnon, 13) will report the dispensability of molybdenum when either ammonia or urea is used as a source of nitrogen.

Previous workers using either fungi (Steinberg, 24; Nicholas *et al.*, 22) or higher plants (Mulder, 18; Hewitt and McCready, 12) concluded that even though the molybdenum requirement was greatly reduced, it could not be completely abolished when ammonium replaced nitrate as the sole source of nitrogen (Meagher, 17; Agarwala, 1). In these species molybdenum has been thought to perform other functions (Hewitt and McCready, 12; Spencer and Wood, 23) in addition to its principal one of catalyzing the reduction of nitrate. However, for the green alga *Chlorella pyrenoidosa*, Walker (26) found a requirement for molybdenum only when nitrate (not urea) was the sole source of nitrogen.

Methods

Apparatus for growing algae

The algae were grown in an apparatus of special design, which has given excellent performance in three years of continuous operation. Because of its general suitability for growing algae, particularly under a variety of nutritional conditions, the apparatus will be described in detail.

The overall appearance of the culture apparatus is shown in Figure 1. The algae were grown with continuous shaking in one-liter Pyrex glass Roux culture bottles laid horizontally on open frame trays. Illumination was provided by fluorescent lights from below. The details of the apparatus are shown diagrammatically in Figure 2.

14 bottles (L) were laid side by side in two rows of seven bottles each on an angle iron tray 101.6 cm. (40 in.) long by 50.8 cm. (20 in.) wide. Each bottle was held in place by 4 rubber-covered vertical pins. The tray was suspended from an angle iron frame by iron straps (O), pivoted on rods in such a manner that the trays could be shaken back and forth with a continuous pendulum-like motion. The distance between the two pivot points on the rocker arm was 12.5 cm. This gave a natural period of oscillation of about 0.7 seconds. The apparatus was driven by a 1/18 horsepower, gear-reduction, motor (Bodine No. B2170—40H). The speed of the motor was adjusted by means of a variable transformer to maintain a shaking rate approximating 85 oscillations per minute. At this rate of shaking, and with a stroke length of 3.8 cm., the algae remained in suspension with very little frothing of the culture medium.

Continuous incident illumination of about 12,000 to 13,000 lux was provided by a bank of eight 48-inch, 40 watt fluorescent tubes (P), mounted on a white-painted board placed 13 cm. below the culture bottles (L). For cooling, an electric fan was

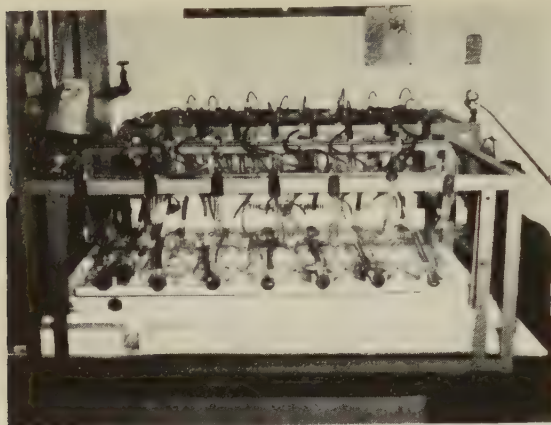


Figure 1. Overall view of algal culture apparatus.

used to blow a stream of air between the fluorescent tubes and the culture bottles. The temperature inside the bottles was maintained no higher than 1.2°C above the ambient temperature which was usually about 25°C . There was less than 1°C difference between the temperatures of bottles in different positions on the shaker.

Each culture bottle contained a maximum of 240 ml. of culture solution. When the bottle was laid in a horizontal position this volume of fluid formed a layer (about 14 mm. thick) shallow enough to prevent spillage into the offset neck of the bottle. The bottles were stoppered with No. 5 rubber stoppers (K), carrying two Pyrex glass tubes, a long L-shaped 5-mm. tube for gas inflow and a short L-shaped 10-mm. tube for gas outflow. Both glass tubes were plugged with non-absorbent cotton (H) to prevent microbial contamination.

The gas, supplied at an approximate rate of 70 ml. per minute per bottle, was a mixture consisting of 5 per cent CO_2 in air. CO_2 from a cylinder (A) flowed through a reducing valve pressure regulator (B), then through a Bernoulli-type gas flow gauge (C) and mixed with air. The air, taken from a compressor air line (D), was held at constant pressure by a reducing valve regulator (E), and a constant head of water (F). The mixture of air and CO_2 flowed through another gas flow gauge (G), then through two cotton filters (H), before entering the manifold (I) to supply the individual bottles. To keep the delivery of the gas mixture to the bottles uniform, a capillary tube (J) was inserted between the manifold and the bottle.

The long L-shaped 5-mm. glass tube for gas inflow was placed to deliver the gas mixture at the far end of the bottle above the algal suspension (Figure 2). Bubbling the gas mixture through the algal suspension produced foaming, particularly in older cultures. With the continuous shaking of bottles, there was no significant difference in growth rates whether the gas mixture was delivered above the algal suspension or bubbled through it.

On leaving the culture bottles, the gas mixture was bubbled through water in a test tube (M) before being released to the atmosphere (N). The bubbling of the effluent gas mixture provided ready visual evidence that each culture bottle was adequately gassed.

The CO_2 content of the gas mixture was checked by a Haldane gas analysis.

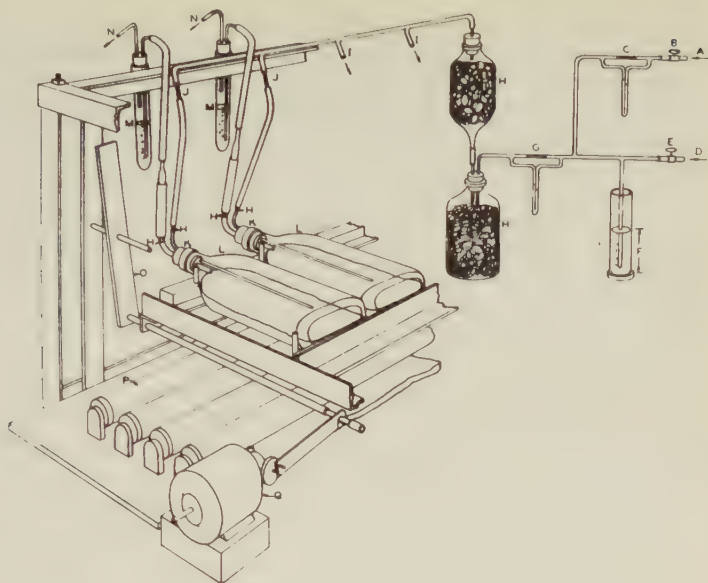


Figure 2. Diagrammatic sketch of algal culture apparatus.

Culture Media

(1) *Preparation of the nutrient solution.* The nutrient solution used in the present investigation was a modification of the one previously described (Arnon and Wesel, 8). Pyrex glass-distilled water was used throughout. The basic nutrient solution consisted of 0.02 *M* KNO_3 , 0.001 *M* MgSO_4 , 0.0005 *M* CaCl_2 and 0.002 *M* of phosphate supplied by a mixture of KH_2PO_4 and K_2HPO_4 (pH 6.7). The components of the basic nutrient solution were purified by the procedures described below. Iron and other micronutrients were used without purification.

The basic nutrient solution was supplemented with the A4 solution supplying boron, manganese, zinc and copper (Arnon, 3), 5 micrograms per liter of vanadium as NH_4VO_3 and 4 milligrams per liter of iron as the ethylene-diamine tetra-acetate complex (Fe-EDTA). Variable amounts of molybdenum (as MoO_3) were added, as indicated, to the individual culture bottles.

The Fe-EDTA solution was prepared by a modification of Jacobson's (14) procedure. 16 grams of EDTA and 10.4 grams of KOH were dissolved in 186 ml. of water. This solution was mixed with an iron solution made by dissolving 13.7 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (low in Mn grade) in 364 ml. of water. Air was bubbled overnight through the mixture to oxidize the iron to the ferric form. 1 ml. of the final solution contained 5 mgs. of Fe and 13 mgs. of K. The final solution which had a pH of approximately 3 showed no signs of precipitation on standing for at least 1 year.

The complete nutrient solution except for phosphate was sterilized by autoclaving at 15–20 lbs. for 20 minutes and allowed to cool. The phosphate solution was autoclaved separately, cooled and added to the nutrient solution. After the addition of phosphate the pH of the nutrient solution was between 6.7–7.0. All operations after autoclaving were carried out under aseptic conditions.

(2) *Purification of nutrient salts.* Stock solutions of KNO_3 , MgSO_4 and CaCl_2 were purified from metal contaminants by a modification of the copper sulfide co-precipitation procedure (Meagher, 17). The procedure used here included an alkaline co-precipitation for the removal of cobalt, nickel, vanadium, etc., followed by an acid co-precipitation step for the removal of molybdenum. In the alkaline co-precipitation step 100 mg. of Cu as $\text{CuSO}_4 \cdot 7 \text{H}_2\text{O}$ and 2 g. CaCO_3 were added to a liter of a molar solution of each of the three salts. After the solutions were brought to a boil, H_2S was bubbled for 20 minutes, the solutions were allowed to cool and the resulting precipitate allowed to settle overnight. The solutions were filtered on folded Whatman No. 12 filter papers which were previously washed with 3 *N* HCl. The filter papers were thoroughly rinsed, prior to use, with glass-distilled water to remove the acid. The removal of the acid was considered adequate when a test of the rinsed filter paper indicated a pH not lower than 4. The residue from the filtration was discarded. The filtrate was prepared for the acid co-precipitation step by being acidified to pH 4 with 1 *N* H_2SO_4 . The CuSO_4 precipitation was then repeated as above except that the CaCO_3 was omitted. Tank nitrogen gas was bubbled for 1–2 hours through the filtrate from the second co-precipitation step to remove any remaining H_2S . The volume of each stock solution was restored to 1 liter with glass-distilled water.

The phosphate stock solution was prepared by purifying a molar solution of KH_2PO_4 by two successive co-precipitation steps as described above except that CaCO_3 was omitted from the first step and the filtrate was not further acidified in the second step. The purified solution was neutralized with solid C.P. KOH to give a $\text{KH}_2\text{PO}_4 : \text{K}_2\text{HPO}_4$ ratio of 1 : 1.

The purity of the stock solutions was tested by the dithizone test (Stout and Arnon, 25). Purification was considered satisfactory if the remaining level of metal impurities corresponded to less than 5×10^{-6} g Zn per liter.

Culture bottles and accessories

As already mentioned, the bottles and the aerators were of Pyrex glass. Following the completion of an experiment all glassware was cleaned, thoroughly rinsed with tap water and allowed to dry. It was then soaked overnight in cleaning solution, again rinsed with tap water and washed with a hot solution of trisodium phosphate (Oakite), followed by a thorough rinsing with distilled water. As a final step prior to use, all glassware was washed with 3 *N* HCl and rinsed 6 times with glass distilled water.

The rubber stoppers were boiled in an Oakite solution and rinsed thoroughly with distilled water. They were then soaked in 3 *N* HCl and thoroughly rinsed with glass-distilled water.

The assembled clean culture bottles were autoclaved prior to use. The requisite amounts of molybdenum were added to each bottle prior to autoclaving.

Inoculum

The algae used for inoculation were grown by consecutive transfers in the nutrient solution (minus molybdenum) as described above except that the nutrient salts were not purified. Molybdenum was not added, in order to produce a low-molybdenum inoculum. Sufficient molybdenum remained as an impurity to satisfy the require-

ments of the algae for a limited time. 2—3 day old, vigorously growing cultures which had attained a cell density of 30,000 to 40,000 cells per mm^3 were used for inoculation. An entire batch of the nutrient solution was inoculated at one time at a rate of 10^7 cells per liter. 240 ml. of the inoculated nutrient solution was poured, under aseptic conditions, into each of the culture bottles.

Growth measurements and chemical determinations

30 to 40 ml. of the culture medium was removed daily under aseptic conditions for growth measurements and chemical determinations. Packed cell volume (fresh weight) was determined as follows: an aliquot of the culture medium was centrifuged to sediment the cells; the supernatant fluid was discarded. The sedimented cells were washed with water and re-centrifuged. The supernatant fluid was discarded and the sedimented cells were resuspended in water and transferred into graduated sedimentation tubes. These were centrifuged in a horizontal position and the volume of the sediment determined. For dry weight determinations the sedimented cells were resuspended in water, transferred into tared beakers, and dried overnight at 80°C . Cell counts were made in a haemocytometer on aliquots of suitable dilution.

Chlorophyll was measured as previously described (Arnon, 4) except that methanol was used to extract the pigment from the cell. Independent tests with the two solvents gave the same chlorophyll values within the limits of experimental error. Chlorophyll (a+b) was determined on algal cells separated from the culture medium by centrifugation. Two successive extractions, carried out in a 60°C water bath, removed chlorophyll completely from the cells. The extracts were centrifuged and the supernatant fluids combined for the measurement of chlorophyll.

Total nitrogen was determined by the micro-Kjeldahl method (9) on aliquots of algal suspension containing between 0.25 to 0.75 mg. N. The algal cells used for nitrogen determinations were sedimented by centrifugation and washed twice by resuspension in distilled water followed by centrifugation.

Results

Effect of molybdenum on growth

The procedures used in the present investigation for purifying the nutrient medium were more effective than those used earlier (Arnon, 5). This is evidenced by the almost complete failure of growth without added molybdenum (Figures 3 and 4; cf. with Figure 1 in reference 5). 10^{-9} g. Mo per liter of nutrient solution (0.001 ppb Mo) was clearly insufficient throughout the entire growth period. 10^{-8} g. Mo per liter was sufficient almost up to the third day but began limiting growth when production of dry cell matter exceeded 2 g/liter. 10^{-7} g. Mo per liter was fully adequate to sustain vigorous growth. No increase in growth was obtained by a ten-fold increase in concentration (10^{-6} g. Mo/l). A further ten-fold increase in concentration (10^{-5} g/l) appeared to be only slightly inhibitory. These and the previous results

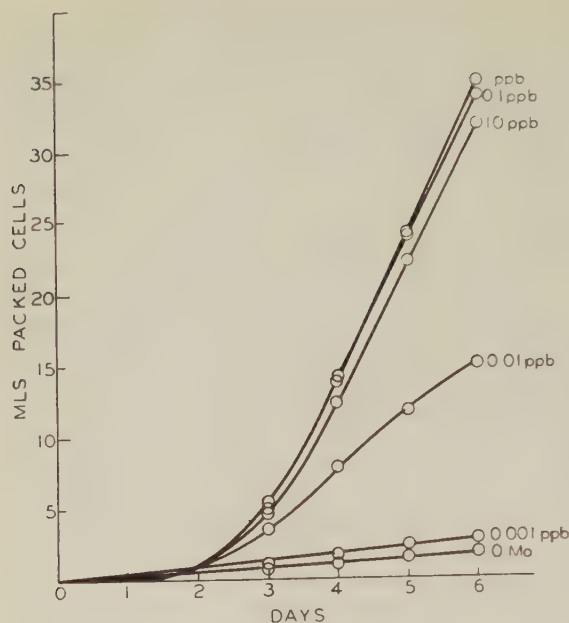


Figure 3. *Effect of molybdenum on the packed cell volume (mls. packed cells per liter of nutrient solution) of Scenedesmus.* (10^{-6} g. Mo per liter of nutrient solution = 1 ppb).

(Arnon, 5) confirm the remarkably great spread, characteristic for molybdenum, between the adequate and toxic levels of supply, as noted in the first experiments on the essentiality of this element for green plants (Arnon and Stout, 7).

The molybdenum requirement of a single cell

The unicellular character of *Scenedesmus* has made it possible to determine the molybdenum requirement in terms of atoms per single cell. The relation between the concentration of molybdenum in the nutrient medium and the number of algal cells is shown in Figure 5. The number of cells in the minus-molybdenum culture served as a blank, since it represented growth at the expense of the molybdenum contained in the inoculum and in the residual impurities of the nutrient medium. By subtracting this blank from the number of cells in the cultures to which molybdenum was added, net cell counts representing growth as a function of molybdenum concentration were obtained.

The values for the molybdenum requirement per cell, given in Table 1, represent maximum figures, since it was assumed that all the molybdenum added to the nutrient solution was used by the algae. The molybdenum requirement per cell was computed at three different levels of molybdenum concentration in the nutrient solution: two representing a restricted, and one

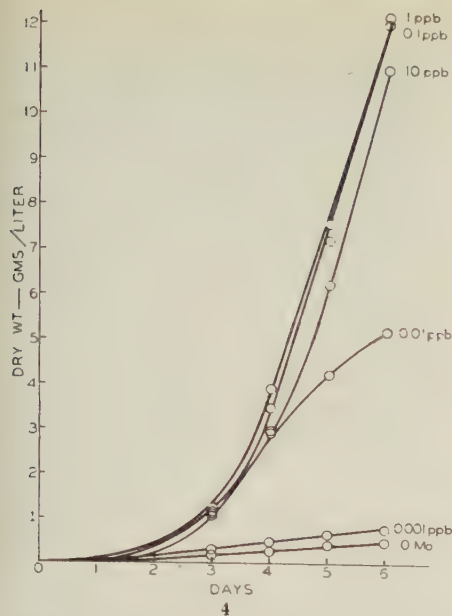


Figure 4. Effect of molybdenum on the dry weight (g. dry weight of cells per liter of nutrient solution) of *Scenedesmus*. (10^{-6} g. Mo per liter of nutrient solution = 1 ppb.)

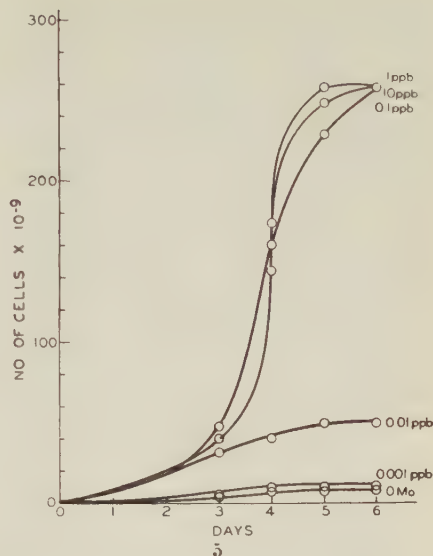


Figure 5. Effect of molybdenum on the number of *Scenedesmus* cells (number of cells $\times 10^{-9}$ per liter of nutrient solution). (10^{-6} g. Mo per liter of nutrient solution = 1 ppb.)

an adequate level of supply (cf. Figure 5). At all three levels the most rapid cell division occurred when the molybdenum supply was above 3000 atoms per cell. In the molybdenum-restricted cultures no cell division occurred when the molybdenum supply dropped to 1500—1700 atoms per cell.

Table 1. Number of Mo atoms per cell in *Scenedesmus*.

Age of culture (days)	Restricted Mo supply				Adequate Mo supply	
	10 ⁻⁹ g/l		10 ⁻⁸ g/l		10 ⁻⁷ g/l	
	Net* cell count $\times 10^{-8}/l$	Atoms Mo per cell**	Net* cell count $\times 10^{-8}/l$	Atoms Mo per cell**	Net* cell count $\times 10^{-8}/l$	Atoms Mo per cell**
0	0.1	630,000	0.1	6,300,000	0.1	63,000,000
3	23	2,700	290	2,200	450	14,000
4	40	1,600	340	1,800	1500	4,200
5	37	1,700	430	1,500	2300	2,700
6	37	1,700	420	1,500	2600	2,400

* Obtained by subtracting the number of cells in the minus Mo culture.

** Computed by the formula: $\frac{\text{gram-atoms Mo}}{\text{net cell count}} \times \text{Avogadro's No.}$

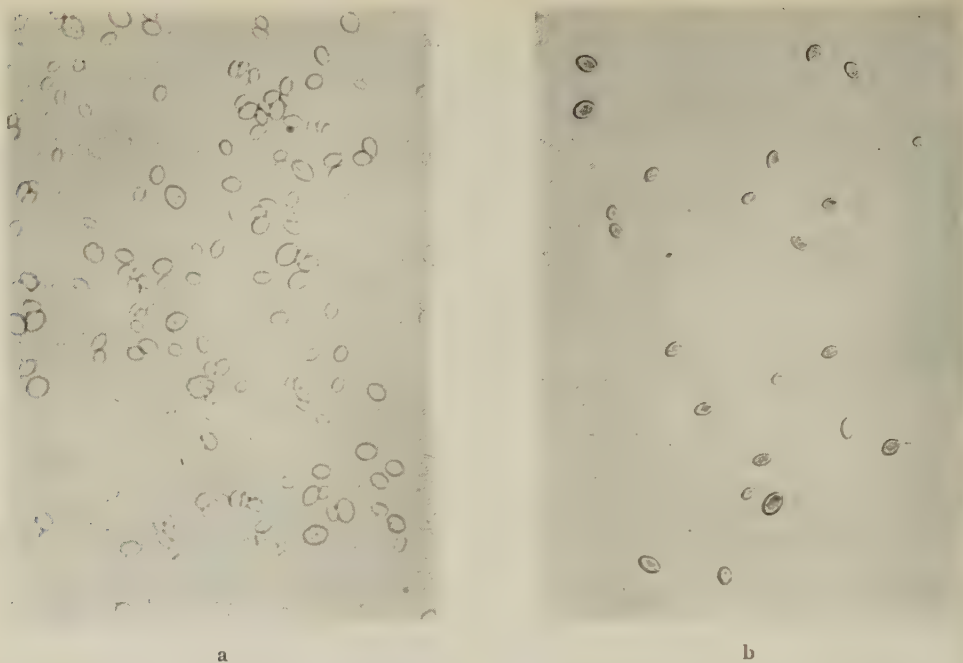


Figure 6. Microphotographs of *Scenedesmus* cells stained with iodine. a. Normal. b. Molybdenum-deficient.

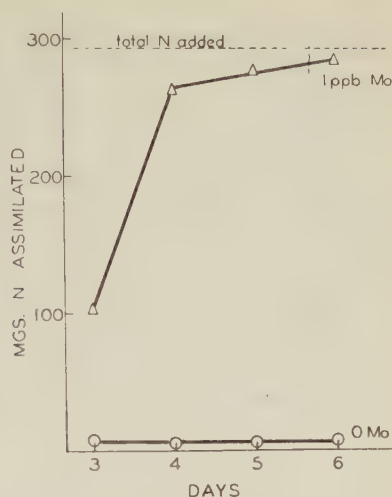
Considering the large errors inherent in this type of computation the quantitative agreement between the molybdenum requirement per cell reported earlier (Arnon, 5) and that found in the different cultures used in the present investigation is considered excellent.

Symptoms of molybdenum deficiency in Scenedesmus

Suspensions of cells from normal and molybdenum-deficient cultures were stained on glass slides with a dilute iodine solution and photographed. The microphotographs are shown in Fig. 6 a and 6 b.

The molybdenum-deficient cells, by contrast with normal cells, stained blue indicating the presence of appreciable quantities of starch. The molybdenum-deficient cells also showed no evidence of cell division. In both these respects the molybdenum-deficient cells were similar in appearance to the nitrogen-deficient *Scenedesmus* cells grown in parallel cultures to which molybdenum was added.

Figure 7. Effect of molybdenum on nitrate nitrogen assimilation by *Scenedesmus*. Ordinate represents mgs. N assimilated by cells contained in one liter of nutrient solution.



Nitrogen assimilation

The nitrogen starvation suggested by the appearance of the molybdenum-deficient cells was borne out by chemical determinations of their nitrogen assimilation. As illustrated in Figure 7 the molybdenum-deficient cells assi-

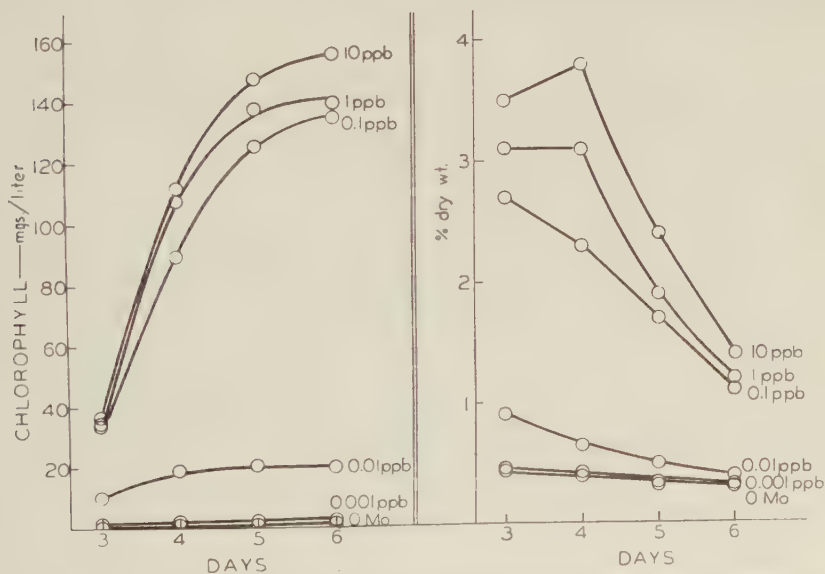


Figure 8. Effect of molybdenum on chlorophyll in nitrate cultures. Left - effect of molybdenum expressed as mgs. of chlorophyll synthesized by cells contained in one liter of nutrient solution; right - effect of molybdenum expressed as per cent of dry weight of *Scenedesmus* cells.

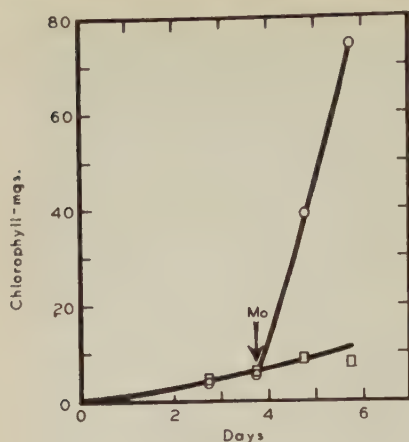


Figure 9. *Resumption of chlorophyll synthesis* (expressed as mgs. chlorophyll synthesized by cells contained in one liter of nutrient solution) upon the addition of 1.3×10^{-5} g. Mo per liter of nutrient solution.

milated only a minute amount of the nitrogen supplied in the nutrient solution. Nitrogen assimilation in the normal cells proceeded vigorously up to the fourth day, when almost all of the nitrogen added to the medium was used up.

Molybdenum in relation to chlorophyll formation

A striking feature previously observed in molybdenum-deficient cells was their low chlorophyll content (Arnon, 5; Loneragan and Arnon, 16). Figure 8 shows that this was also the case in the present investigation. The relation between molybdenum and chlorophyll is evident whether it is expressed on a cell content basis (per cent dry weight) or as milligrams of chlorophyll formed per liter of culture medium.

Chlorophyll content increased with increasing molybdenum supply within the range investigated. This is especially interesting at the highest concentration of molybdenum (10^{-5} g./l) which, though associated with the highest chlorophyll synthesis, produced no further increase in growth (Figures 3 and 4). For any one level of molybdenum supply the chlorophyll content of cells progressively decreased as growth increased after the fourth day.

The addition of molybdenum to molybdenum-deficient cells resulted in a prompt and sharp increase in chlorophyll synthesis (Fig. 9). It was previously shown (Arnon, 5) that this increase in chlorophyll coincided with an increase in growth (cf. Figures 2 and 3 in reference 5).

Discussion

The results obtained in these and many other similar experiments in which the same purification methods of the nutrient medium were used leave little

doubt that, when nitrate is the sole source of nitrogen, molybdenum is indispensable for the growth of *Scenedesmus*. As reported earlier (Arnon, 5), the requirement for molybdenum was found to be specific. Molybdenum deficiency was corrected by the addition of this element but not by that of 22 others (the remaining elements in the B7 and C13 solutions; cf. Arnon, 3).

The quantitative requirement for molybdenum by *Scenedesmus* cells was found to be extremely low. In terms of atoms per cell, good growth occurred with 3000 atoms per cell. When the supply was reduced to 1500-1700 atoms per cell, no cell division took place. These findings invite comparison with those reported by Burk for *Azotobacter* (10). He found that *Azotobacter* required 10000 atoms of Mo for optimum nitrogen fixation. Taking the average diameter of a *Scenedesmus* cell at 12 and that of *Azotobacter* as 2 microns (10) one arrives at the ratio of the cell volumes of *Scenedesmus* to *Azotobacter* as 216 to 1 (ratio of the diameters cubed). This suggests that on a unit cell volume basis, the optimum concentration of molybdenum in *Azotobacter* is approximately 700 times as great as in *Scenedesmus*.

If the quantitative requirement for molybdenum by *Scenedesmus* is expressed in terms of concentration of this element in the nutrient solution, remarkably good agreement is found with the low values reported by Agarwala and Hewitt (2) for cauliflower. The supply levels at which molybdenum deficiency could be shown were 10^{-9} to 10^{-8} g. Mo per liter for *Scenedesmus* as against 5×10^{-9} to 5×10^{-8} g. Mo per liter of nutrient solution for cauliflower (2).

The extremely low quantitative requirement for molybdenum argues strongly for the catalytic function of this element in the metabolism of the cell. The failure of molybdenum-deficient cells to assimilate nitrate nitrogen supports the view that in *Scenedesmus*, as in *Neurospora* (20, 21), molybdenum acts as the specific metal component of nitrate reductase.

Less obvious is the reason for the marked association of molybdenum with chlorophyll synthesis. Using tomato as a test plant, Hewitt and McCready found (12), as we did with *Scenedesmus*, a marked reduction in the chlorophyll content of the Mo-deficient plants when nitrate was a source of nitrogen. With ammonium nitrogen however, they found the chlorophyll content of Mo-deficient plants to be actually higher than in the controls. In the light of the data presented elsewhere (Ichioka and Arnon, 13) it seems probable that the molybdenum effect on chlorophyll is indirect and is related to the assimilation of nitrate-nitrogen.

Summary

In a purified nutrient medium in which nitrate served as the sole source of nitrogen, molybdenum was essential for growth and cell division of the green alga, *Scenedesmus obliquus*.

The requirement of molybdenum was found to be extremely low: to demonstrate marked deficiency the concentration in the nutrient solution had to be reduced to a range between 10^{-9} and 10^{-8} g. Mo per liter of nutrient solution.

To assure good growth ca. 3000 atoms of molybdenum per cell were required. No cell division occurred when the supply of molybdenum was reduced to 1500-1700 atoms per cell.

Molybdenum deficient cells failed to assimilate nitrate nitrogen. Microscopic examination revealed the accumulation of starch and a general appearance similar to that of nitrogen-starved cells.

Molybdenum deficiency was associated with a marked reduction in chlorophyll content. The chlorophyll content increased with increasing molybdenum concentration in the nutrient solution.

This work was aided in part by a grant from the Climax Molybdenum Company, New York, N. Y. We are indebted to Dr. C. M. Johnson for his many helpful suggestions on the purification of nutrient salts.

References

1. Agarwala, S. C.: Relation of nitrogen supply to the molybdenum requirement of cauliflower grown in sand culture. — *Nature* 169: 1099, 1952.
2. — & Hewitt, E. J.: Molybdenum as a plant nutrient III. The interrelationships of molybdenum and nitrate supply in the growth and molybdenum content of cauliflower plants grown in sand culture. — *Jour. Hort. Sci.* 29: 278, 1954.
3. Arnon, D. I.: Microelements in culture-solution experiments with higher plants. — *Amer. J. Bot.* 25: 322, 1938.
4. — Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. — *Plant Physiol.* 24: 1, 1949.
5. — Some recent advances in the study of essential micronutrients for green plants. — 8e Congres Intern. Botan. Paris, Rapports & Communicat. Sect. 11: 73, 1954.
6. — Fujiwara, A., Wessel, G. & Woolley, J. T.: Molybdenum in the nutrition of *Scenedesmus*. — Paper presented before Amer. Soc. Plant. Physiol. Madison, Wisconsin, September 1953.
7. — & Stout, P. R.: Molybdenum as an essential element for higher plants. — *Plant Physiol.* 14: 599, 1939.
8. — & Wessel, G.: Vanadium as an essential element for green plants. — *Nature* 172: 1039, 1953.
9. Association of official agricultural chemists. Official methods of analysis. 7th Ed. 910 pp. Washington, D. C. 1950.
10. Burk, D.: Azotase and nitrogenase in *Azotobacter*. — *Ergebn. Enzymforsch.* 3: 23, 1934.
11. Hewitt, E. J. & Jones, E. W.: The production of molybdenum deficiency in plants in sand culture with special reference to tomato and brassica crops. — *J. Pom. Hort. Sci.* 23: 254, 1947.
12. — & McCready, C. C.: Relation of nitrogen supply to the molybdenum requirement of tomato plants grown in sand culture. — *Nature* 174: 186, 1954.

13. Ichioka, P. S. & Arnon, D. I.: Molybdenum in relation to nitrogen metabolism. II Assimilation of ammonia and urea without molybdenum by *Scenedesmus*. — *Physiol. Plant.* 8: 552. 1955.
14. Jacobson, L.: Maintenance of iron supply in nutrient solutions by a single addition of ferric potassium ethylenediamine tetra-acetate. — *Plant Physiol.* 26: 411. 1951.
15. Ketchum, B. H.: Mineral nutrition of phytoplankton. — *Ann. Rev. Plant Physiol.* 5: 55. 1954.
16. Loneragan, J. F. & Arnon, D. I.: Molybdenum in the growth and metabolism of *Chlorella*. — *Nature* 174: 459. 1954.
17. Meagher, W. R.: Molybdenum nutrition of the higher plant as revealed by nitrogen metabolites. — Doctoral thesis. Univ. of Calif. Berkeley, Calif. 1952.
18. Mulder, E. G.: Importance of molybdenum in the nitrogen metabolism of micro-organisms and higher plants. — *Plant & Soil* 1: 94. 1948.
19. Myers, J.: Physiology of the algae. — *Ann. Rev. Microb.* 5: 157. 1951.
20. Nicholas, D. J. D. & Nason, A.: Molybdenum and nitrate reductase. II Molybdenum as a constituent of nitrate reductase. — *J. Biol. Chem.* 207: 353. 1954.
21. — — Mechanism of action of nitrate reductase from *Neurospora*. — *J. Biol. Chem.* 211: 183. 1954.
22. — & McElroy, W. D.: Molybdenum and nitrate reductase. I Effect of molybdenum deficiency on the *Neurospora* enzyme. — *J. Biol. Chem.* 207: 341. 1954.
23. Spencer, D. & Wood, J. G.: The role of molybdenum in nitrate reduction in higher plants. — *Aust. J. Biol. Sci.* 7: 425. 1954.
24. Steinberg, R. A.: Role of molybdenum in the utilization of ammonium and nitrate nitrogen by *Aspergillus niger*. — *J. Agr. Res.* 55: 891. 1937.
25. Stout, P. R. & Arnon, D. I.: Experimental methods for the study of the role of copper, manganese and zinc in the nutrition of higher plants. — *Amer. J. Bot.* 26: 144. 1939.
26. Walker, J. B.: Inorganic micronutrient requirements of *Chlorella*. I Requirements for calcium (or strontium), copper and molybdenum. — *Arch. Biochem. Biophys.* 46: 1. 1953.
27. Wilson, P. W. & Burris, R. H.: Biological nitrogen fixation — a reappraisal. — *Ann. Rev. Microbiol.* 7: 415. 1953.

Molybdenum in Relation to Nitrogen Metabolism

II. Assimilation of Ammonia and Urea without Molybdenum by *Scenedesmus*

By

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(Received April 22, 1955)

Introduction

The preceding paper (2) reported the essentiality of molybdenum for *Scenedesmus obliquus* (Strain D 3) when nitrate was used as the sole source of nitrogen. In the current investigation the requirement for molybdenum has been abolished when nitrate was replaced by either urea or ammonia. The results suggest that in *Scenedesmus* the function of molybdenum is limited to nitrate reduction. This seems to provide the first instance in the nutrition of green plants in which the essentiality of an inorganic element is linked with only one enzyme system. The bypassing of the specific »metabolic block» caused by the deficiency of molybdenum rendered this essential element dispensable. With other organisms the substitution of a reduced form of nitrogen for nitrate has not abolished the requirement for molybdenum (literature cited in 2).

Methods

The apparatus for growing algae, culture bottles, methods of inoculation, growth measurements and chemical determinations were the same as those described in the preceding paper (2).

Except for the different nitrogen regimen, the nutrient solutions were also the same as those previously described (2). 0.0005 M Ca (NO₃)₂ was used as

a source of calcium in all cultures instead of CaCl_2 (2). The small amount of nitrogen contributed by the $\text{Ca}(\text{NO}_3)_2$ was of little quantitative importance relative to the large requirements of this element for growth. Molybdenum was added to the plus molybdenum cultures at a concentration of 10^{-6} g. Mo per liter of nutrient solution (1 ppb.).

The purification and preparation of the nutrient solutions were the same as described earlier (2). Urea and $(\text{NH}_4)_2\text{CO}_3$ solutions were sterilized by filtration and added to the autoclaved nutrient solution. $\text{Ca}(\text{NO}_3)_2$ was as free from molybdenum as CaCl_2 (2). Urea was purified by two recrystallizations from H_2O . A molar stock solution of the purified urea was used in making nutrient solutions.

For the preparation of purified ammonium carbonate, NH_4OH (C. P. grade) was distilled into water. Tank CO_2 gas was bubbled into the distillate until the pH fell to 8. The molarity of the resultant ammonium carbonate solution was determined by titration with 0.01 N H_2SO_4 , with methyl red-bromocresol green as an indicator.

Results

I. Normal N supply

Effect of Mo and N source on growth and chlorophyll. The cultures were divided into three series, each supplied at the start with 20 millequivalents of nitrogen per liter from one of three sources: potassium nitrate, urea or ammonium carbonate. Within each series molybdenum was added to half of the cultures.

Figure 1 shows that in the nitrate series no growth occurred without added

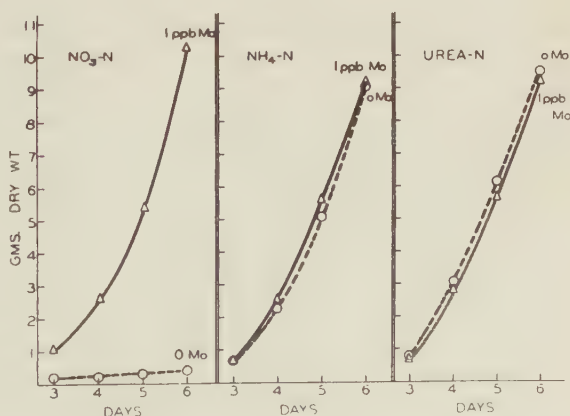


Figure 1. *Effect of molybdenum on growth of Scenedesmus supplied with nitrate, ammonia, or urea nitrogen.* (Ordinate represents grams dry weight of cells per liter of nutrient solution.)

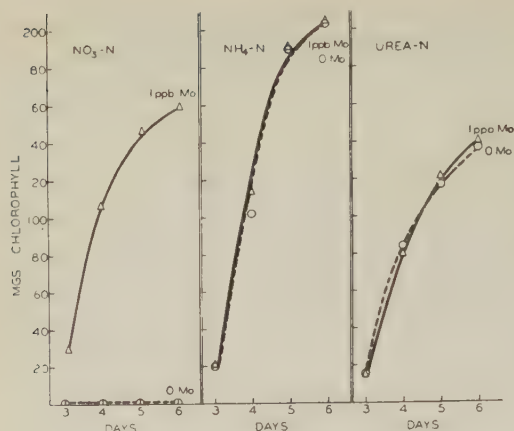


Figure 2. *Effect of molybdenum on chlorophyll formation by Scenedesmus supplied with nitrate, ammonia, or urea nitrogen.* (Ordinate represents milligrams of chlorophyll synthesized by cells contained in 1 liter of nutrient solution.)

molybdenum (cf. 2). By contrast, the addition of molybdenum had no effect on growth with either urea or ammonia nitrogen. The effect of molybdenum on chlorophyll formation (Figure 2) paralleled the effect of this element on growth. Without added molybdenum, chlorophyll synthesis was completely arrested in the nitrate series but not in the urea or ammonia series.

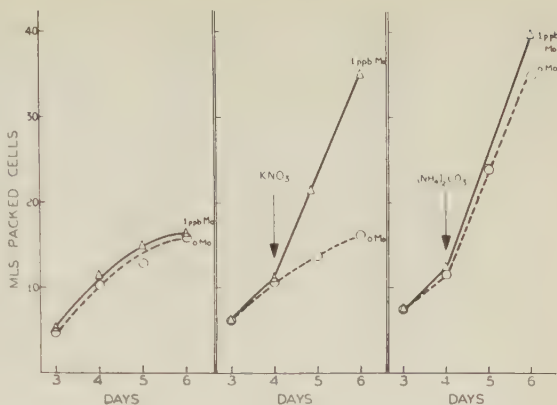
The conclusion was drawn from these experiments that molybdenum is required solely for nitrate reduction. The effect of molybdenum on chlorophyll was observed only in the nitrate cultures and could be explained by its effect on nitrogen metabolism.

The dispensability of molybdenum when either ammonia or urea were used as sources of nitrogen was further tested as described in the following section.

II. Restricted N supply

Effect of Mo and N source on growth. The cultures in these experiments were divided into two series, one supplied with urea and the other with ammonium carbonate. The general design of the experiments was to supply a restricted amount of ammonia or urea (0.035 M) to plus- and minus-molybdenum cultures in each series. On the fourth day, when the nitrogen supply was exhausted, each series was subdivided into 3 groups. One group served as a control and received no further addition of nitrogen; the second group received an addition of KNO₃. If molybdenum were present as a contaminant in either the urea or ammonia media it would have permitted the assimilation of the added nitrate in the cultures with no added molybdenum. No such effect was observed, indicating the purity of the urea or ammonia used. The third group of cultures with a restricted nitrogen supply received an addition

Figure 3. *Effect of molybdenum on utilization of supplementary nitrogen added to Scenedesmus grown with a restricted supply of ammonium carbonate. At times indicated by arrow 20 milliequivalents of nitrogen were added. Left - control; center - potassium nitrate; right - ammonium carbonate. (Ordinate represents mls. packed cells per liter of nutrient solution.)*

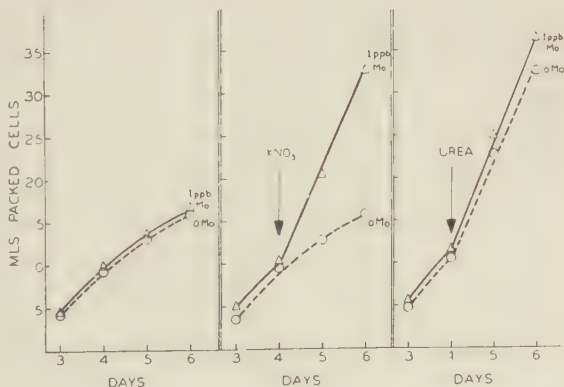


of either urea or ammonium carbonate. The addition of supplementary nitrogen on the fourth day consisted of 20 milliequivalents of N per liter of nutrient solution.

Figures 3 and 4 illustrate the effect of molybdenum and different sources of nitrogen on growth as measured by packed cell volume. When either urea or ammonia was used as the initial nitrogen source, molybdenum had no effect on growth. On the fourth day, when the then limiting nitrogen supply was augmented by additions of the various nitrogen salts, molybdenum exercised a differential effect on their utilization. Potassium nitrate gave a prompt and sharp increase in growth only in the presence of molybdenum, whereas urea and ammonia were utilized at about the same rates with or without molybdenum.

A differential effect of molybdenum on growth with nitrate versus that with ammonia and urea is seen in Figures 5 and 6, which represent dry

Figure 4. *Effect of molybdenum on utilization of supplementary nitrogen added to Scenedesmus grown with a restricted supply of urea. At times indicated by arrow 20 milliequivalents of nitrogen were added. Left - control; center - potassium nitrate; right - urea. (Ordinate represents mls. packed cells per liter of nutrient solution.)*



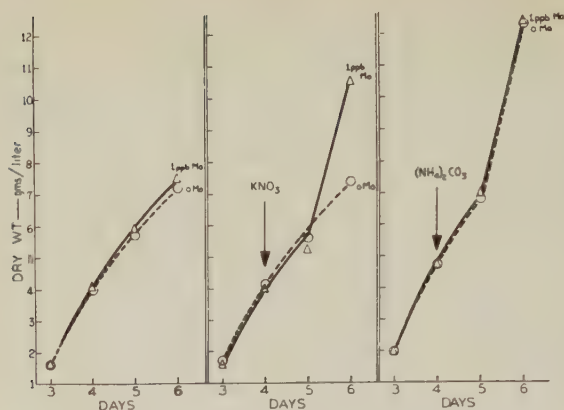


Figure 5. Effect of molybdenum on utilization of supplementary nitrogen added to *Scenedesmus* grown with a restricted supply of ammonium carbonate. At times indicated by arrow 20 milliequivalents of nitrogen were added. Left - control; center - potassium nitrate; right - ammonium carbonate. (Ordinate represents grams dry wt. of cells per liter of nutrient solution.)

weight. The restriction in dry weight accumulation, whether resulting from a limiting nitrogen supply or a molybdenum deficiency, was less pronounced than the restriction in cell volume. This is probably due to the continuing accumulation of starch under conditions of nitrogen starvation. Starch accumulation in the deficient cultures would also explain the apparent lag in response to added nitrogen, as measured by dry weight (compare with Figure 3).

Effect of molybdenum on assimilation of different sources of nitrogen

The dispensability of molybdenum for the assimilation of urea and ammonia and its indispensability for the assimilation of nitrate nitrogen is shown in Figures 7 and 8. These direct data on nitrogen assimilation provide a sharper indication of the role of molybdenum than growth measurements by

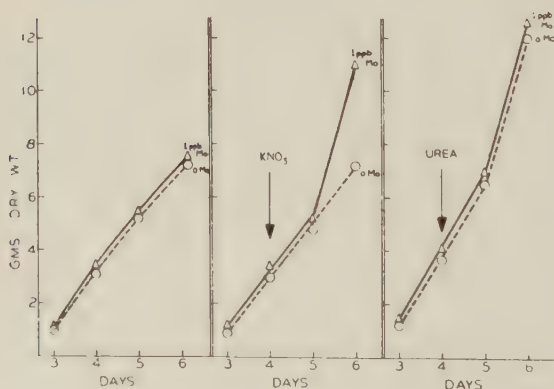


Figure 6. Effect of molybdenum on utilization of supplementary nitrogen added to *Scenedesmus* grown with a restricted supply of urea. At times indicated by arrow 20 milliequivalents of nitrogen were added. Left - control; center - potassium nitrate; right - urea. (Ordinate represents grams dry wt. of cells per liter of nutrient solution.)

Figure 7. Effect of molybdenum on assimilation of supplementary nitrogen by *Scenedesmus* grown with a restricted supply of ammonium carbonate. At times indicated by arrow 20 milliequivalents of nitrogen were added. Left - control; center - potassium nitrate; right - ammonium carbonate. (Ordinate represents mgs. nitrogen assimilated by cells contained in one liter of nutrient solution.)

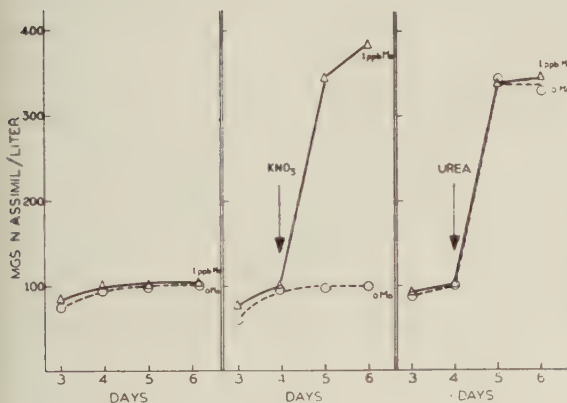
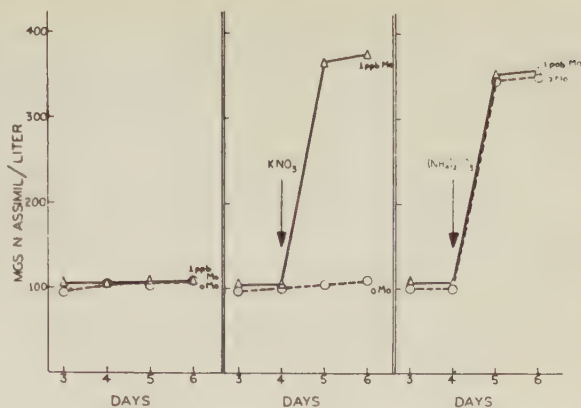
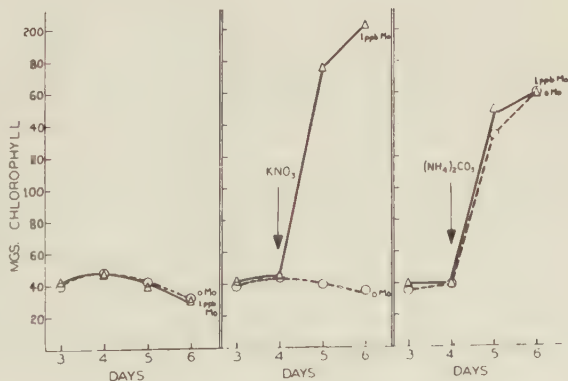


Figure 8. Effect of molybdenum on assimilation of supplementary nitrogen by *Scenedesmus* grown with a restricted supply of urea. At times indicated by arrow 20 milliequivalents of nitrogen were added. Left - control; center - potassium nitrate; right - urea. (Ordinate represents mgs. nitrogen assimilated by cells contained in one liter of nutrient solution.)

Figure 9. Effect of molybdenum on chlorophyll formation by *Scenedesmus* grown with a restricted supply of ammonium carbonate. At times indicated by arrow 20 milliequivalents of nitrogen were added. Left - control; center - potassium nitrate; right - ammonium carbonate. (Ordinate represents mgs. chlorophyll synthesized by cells contained in one liter of nutrient solution.)



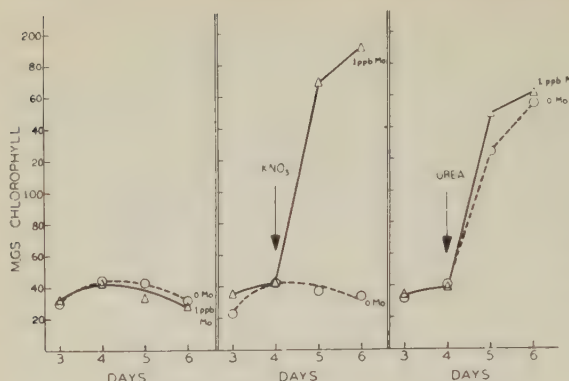


Figure 10. Effect of molybdenum on chlorophyll formation by *Scenedesmus* grown with a restricted supply of urea. At times indicated by arrow 20 milliequivalents of nitrogen were added. Left - control; center - potassium nitrate; right - urea. (Ordinate represents mgs. chlorophyll synthesized by cells contained in one liter of nutrient solution.)

dry or fresh weight. Limited increments in dry or fresh weight, resulting from the accumulation of carbohydrates, are conceivable under conditions of nitrogen starvation.

Effect of molybdenum and nitrogen source on chlorophyll

The effect of molybdenum and different sources of nitrogen on chlorophyll formation is shown in Figures 9 and 10. The data show a parallelism between the effects of molybdenum on nitrogen assimilation and chlorophyll synthesis. Molybdenum had no significant effect on chlorophyll formation when ammonia or urea served as a source of nitrogen. Molybdenum deficiency resulted in suppression of chlorophyll synthesis when nitrate was the source of nitrogen.

Discussion

The absence of any measurable molybdenum requirement when urea or ammonia were used as sources of nitrogen for *Scenedesmus*, supports the conclusion anticipated in the previous paper (2), that the function of molybdenum in this organism is confined to nitrate reduction. The relation of molybdenum to nitrite assimilation in *Scenedesmus* is currently under investigation.

The purified ammonium carbonate and urea used made it unlikely that these two sources of reduced nitrogen contained significant impurities of molybdenum. This was particularly true of the ammonium carbonate which was prepared from distilled ammonia and CO₂ gas. With restricted nitrogen supply it was confirmed that these two compounds were free from detectable molybdenum contamination. In the restricted nitrogen series, all cultures

received initially ammonia or urea nitrogen and would have thus acquired whatever residual molybdenum contamination was left in these nitrogen sources. Yet when nitrate nitrogen was added later it was assimilated only by the plus molybdenum cultures. No effect of molybdenum was observed with either urea or ammonia.

The striking correlation between molybdenum deficiency and failure of chlorophyll synthesis (cf. 2) has also disappeared when nitrate was replaced by ammonia or urea nitrogen. This suggests that the observed effect of molybdenum on chlorophyll synthesis is indirect, and operates only when nitrate reduction is blocked. This sensitive relation between nitrogen metabolism and chlorophyll formation need not be related to the nitrogen content (6 per cent of the molecular weight) of the pigment itself. More likely, the sensitive relation between nitrogen assimilation and chlorophyll formation stems from the dependence of chlorophyll synthesis and function on association with specific proteins. When supplied with nitrate nitrogen molybdenum-deficient cells are unable to assimilate it and thus become, physiologically speaking, nitrogen-starved cells without any capacity for protein synthesis.

Despite the distinct correlation between molybdenum and nitrate assimilation in *Scenedesmus* it appears certain that the role of molybdenum in the nitrogen metabolism of green plants in general is not confined to the reduction of nitrate. In support of this conclusion we may cite the molybdenum requirement of blue-green algae for nitrogen fixation. The molybdenum requirement is specific and cannot be replaced by vanadium (Allen and Arnon, 1). There is no valid evidence that the fixation of molecular nitrogen involves the formation and subsequent reduction of nitrate (Wilson and Burris, 4). Of special interest in this connection is the recently found (Shug et al., 3) association of molybdenum with hydrogenase from the anaerobic nitrogen-fixing bacterium *Clostridium pasteurianum*.

Summary

Scenedesmus was grown with and without added molybdenum in purified nutrient solutions to which nitrogen was supplied by one of three sources: potassium nitrate, urea or ammonium carbonate.

Molybdenum was found to be essential for growth, nitrogen assimilation and chlorophyll formation only in the nitrate cultures. The requirement for molybdenum was abolished when nitrate was replaced by either urea or ammonium carbonate.

The indispensability of molybdenum for the assimilation of nitrate nitrogen and the dispensability of this element for the assimilation of ammonia or

urea nitrogen was observed by two experimental approaches. In one series of experiments an initially adequate supply of nitrate, urea or ammonia nitrogen was added at the start. In another series of experiments, all cultures were initially given a restricted amount of nitrogen either as ammonia or urea. The restricted nitrogen supply was later supplemented by additions of nitrate, ammonia or urea nitrogen.

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References

1. Allen, M. B. and Arnon, D. I.: Studies on nitrogen-fixing blue-green algae. — I. Growth and nitrogen fixation by *Anabaena cylindrica* Lemm. — *Plant Physiol.* (Manuscript submitted for publication).
2. Arnon, D. I., Ichioka, P. S., Wessel, G., Fujiwara, A., and Wolley, J. T.: Molybdenum in relation to nitrogen metabolism. — I. Assimilation of nitrate nitrogen by *Scenedesmus*. — *Physiol. Plant.* 8: 538. 1955.
3. Shug, A. L., Wilson, P. W., Green, D. E. and Mahler, M. R.: The role of molybdenum and flavin in hydrogenase. — *Journ. Amer. Chem. Soc.* 76: 3355. 1954.
4. Wilson, P. W. and Burris, R. H.: The mechanism of biological nitrogen fixation — *Bacter. Rev.* 11: 41. 1947.

The Respiratory Quotient in Different Parts of Wheat Roots in Relation to Growth

By

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Introduction

The literature concerning the respiratory quotient ($R.Q. = CO_2/O_2$) in higher plants has recently been reviewed by James. Earlier a thorough theoretical treatment of the R.Q. and its connection with the total metabolism of the cells has been carried out by Tamiya based on experiments with *Aspergillus oryzae*. The R.Q. reflects the oxidation and reduction processes occurring in the living organism, and Tamiya showed that it is possible to calculate the R.Q. if the combustion quotients, i.e., the oxidation levels, of the substrate and of the substances formed are known.

This is the consequence of the fact that an oxidation process is always coupled to a reduction process. Thus the oxidation of a certain amount of an organic compound to carbon dioxide and water is accompanied by the reduction of an equivalent amount of other substances. If the substance reduced is molecular oxygen, as is the case to a large extent in aerobic respiration, the R.Q. will be unity if the substance oxidized is a carbohydrate, above unity if the substance is richer in oxygen than carbohydrates, and below unity if the substance is poorer in oxygen than carbohydrates. But besides oxygen even other substances, usually metabolic intermediates, may serve as hydrogen acceptors, and this will of course affect the R.Q. value. This is the case in fermentation and — in the normal aerobic metabolism — in the biosynthesis of lipids, proteins, and other substances more reduced than the starting material which usually is carbohydrates. In plants with nitrate as a nitrogen source the reduction of the nitrate will also affect the R.Q. as has been thoroughly investigated by Yamagata. Such reduction processes increase the R.Q. On the other hand, oxidation processes not leading to evolution of carbon dioxide will have an influence on the R.Q. in the opposite direction.

Thus the R.Q. is determined not only by the nature of the substrates broken down but of the total metabolism of the cell. In most plant tissues with adequate oxygen supply the R.Q. is close to unity, corresponding to a complete oxidation of carbohydrates (cf. James). In growing tissues, however, an influence on the R.Q. from the synthesis of new cell matter taking place there is to be expected, provided that the average oxidation levels of the substrate and the newly formed substances are not the same. Ruhland and Ramshorn reported for embryonic tissue R.Q. values considerably exceeding unity. Other investigators, however, have obtained R.Q. values deviating only slightly from unity even for embryonic tissue. Thus Wanner states that the R.Q. even in the meristematic part of excised wheat roots is about unity. Baldovinos obtained R.Q. values for different zones of young *Zea* roots under different conditions varying between 0.88 and 1.14. Kandler (1950), who cultivated excised *Zea* roots on a glucose medium with nitrate as a nitrogen source, found during the first four days R.Q. values above unity (up to 1.3). He also investigated the effect of the oxygen tension on the R.Q. of root tips and obtained evidence that the high R.Q. values of Ruhland and Ramshorn were due to fermentation processes caused by lack of oxygen. In a later paper Kandler (1953) has reported R.Q. values of 1.1—1.2 for isolated embryos and roots of *Zea* cultivated with glucose as the substrate. His values show that the magnitude of the R.Q. is dependent on how much of the assimilated glucose is used for the formation of new cell substances.

The present investigation deals with the respiration and the R.Q. in different parts of young wheat roots. In order to study the influence of growth on these phenomena the experiments have been carried out with roots which have grown with different growth rates. The growth has been regulated by supplying, on the one hand, β -indolylacetic acid (IAA) in growth-inhibitory concentrations, on the other hand, α -indolylisobutyric acid (IIBA), a growth-stimulating substance for roots. The measurements have been carried out both without and with the supply of substrate (glucose) to the root segments. Of particular interest in this connexion is the fact that the difference between the oxygen consumption in the first case (endogenous respiration) and in the latter one varies with the distance from the tip. This variation corresponds to the variation of the R. Q.

Methods

The experimental material was roots of wheat seedlings (Weibulls orig. *»Eroica»*). These were cultured under sterile conditions according to a method earlier described, and the methods used coincide also in other respects with those described by Eliasson.

After culturing for two days in sterile nutrient solution the lengths of the roots were measured and they were cut into segments of desired lengths with razors. Before and after the respiration determinations the segments were weighed on a torsion balance after rapid surface drying on filter paper. As a measure of the fresh weight of a sample the mean value of these two weighings was used. The fresh weight determined in this way turned out to be the most exact measure of the amount of roots in each sample obtainable in a simple way.

The gaseous exchange was measured at 25° C in a Warburg apparatus, the carbon dioxide output being determined according to the »direct method» described by Umbreit et al. In the Warburg flasks the root segments were suspended in 2.0 ml nutrient solution of the same composition as the growth solution (see Eliasson) but with the pH adjusted to 4.5. The respiratory determinations with the exception of the time experiments (figures 1 and 2) were started three hours after the excision of the roots. The endogenous respiration was determined for three hours with readings every 30 minutes. Then glucose solution was added from the sidearm of the flask, the glucose concentration of the main solution being 0.05 *M*. After half an hour for calibration the respiration was determined for another two hours. The number of segments in each flask varied from 40 to 120 owing to differences in their length and respiratory intensity.

Results

The experimental results are presented mainly in graphical form in figures 1—6. Each curve is based on two or three series of experiments. The differences between the values obtained for the same material in different experiments only rarely exceed 20 per cent (see table 1).

Figures 1 and 2 show the time course of the gas exchange and of the R.Q. without and with glucose supply. In the latter case the root segments have been placed in the glucose solution immediately after the excision. The determinations were started half an hour after the excision. These experiments have been carried out only with roots grown without supplied growth substances. Segments from three different zones of the root have been used. Segment A, 0—4 mm from the tip, consists of the meristem and the main part of the extending zone (cf. Eliasson). Segment B, 4—10 mm from the tip, consists of the rest of the extending zone (about 1 mm) and the beginning of the differentiation zone. Segment C is taken 20—30 mm from the tip.

The endogenous respiration is characterized by a strong decrease during the course of the experiment, while the gas exchange in presence of glucose is, on the whole, constant. This indicates that the decrease is due to lack of respirable materials (cf. James). This is consistent with the fact that the R.Q. decreases in the absence of glucose. This decrease may be explained as a consequence of the consumption of the normal respiratory substances, soluble carbohydrates, and decomposition of protoplasmic constituents as lipids and proteins. In the growing parts of the root the cessation of the reduction pro-

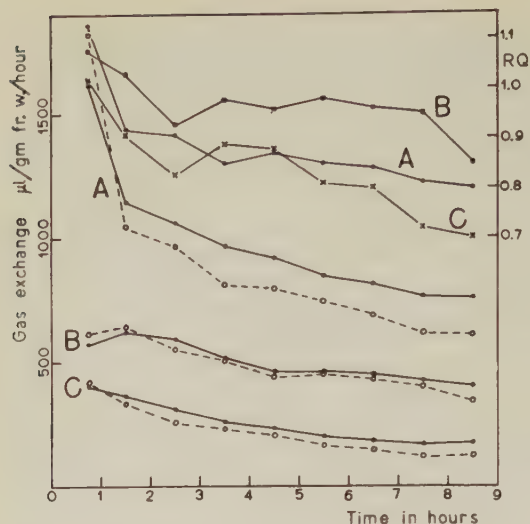


Figure 1. The time course of the endogenous O_2 uptake (solid lines), CO_2 output (broken lines), and R.Q. (solid lines). The positions in the root of the segments used have been: A 0—4 mm, B 4—10 mm, and C 20—30 mm from the tip. The roots have been excised at zero time. Each point represents the mean value of two experimental figures.

cesses involved in the synthesis of new cell substances will also have a decreasing effect on the R.Q. An interesting point is that the R.Q. values at the beginning of the experiments are greater than unity (1.08—1.15) in segments A and B but about unity in mature tissue. It is probable that these first determinations are close to the values of the intact root. The fact that the R.Q. is greater than unity for the two first segments in the beginning of the experiments and for all the segments in the later part of the experiments with glucose is obviously due to the synthesis of substances poorer in oxygen than carbohydrates.

The experiments indicate some important divergences in the respiration between different parts of the root regarding the following points:

1. There is a strong decrease in the gas exchange per unit fresh weight from the tip towards the basal part of the root.
2. The effect on the respiration of the glucose supply is not so great in segment B as it is in the segments A and C.
3. The decrease of the R.Q. is much smaller in segment B than in the segments A and C.

The variation of these properties with the distance from the tip has been subjected to more exhaustive investigations. Besides roots with normal growth rate, roots with inhibited or stimulated growth have been used in these experiments, the inhibition being caused by IAA and the stimulation by IIBA. The length of the segments and their situation in the roots have been from the tip: I, 0—2 mm; II, 2—4 mm; III, 4—7 mm; IV, 7—10 mm; V, 10—20 mm; VI, 20—30 mm. Segment I consists mainly of meristematic tissue (cf. Elias-

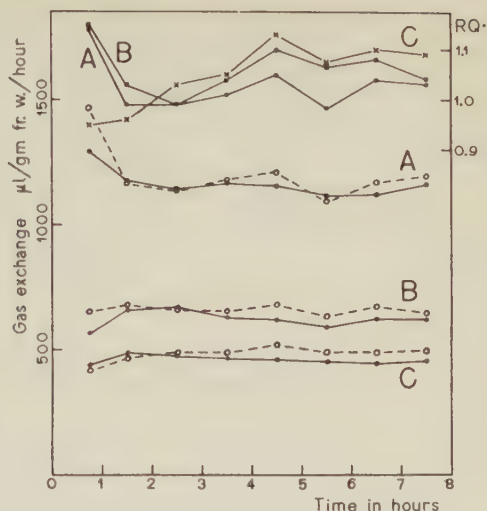


Figure 2: The time course of the respiration in 0.05 M glucose solution. In other respects the experiments and the designations correspond to those in figure 1.

son). The length of the extension zone varies with the growth rate. This zone thus occupies in the IAA roots the main part of segment II, in roots with normal growth segment II and the first half of segment III, and in IIBA roots the segments II and III.

Table 1. Gas exchange for different segments of wheat roots per gm fresh weight and hour. The seedlings cultured for two days in sterile growth solutions at 22° C in dark. Growth in length 4.1 cm. The endogenous respiration was determined 3–6 hours after excision and then glucose was added.

Segment (mm from the tip)	Endogenous respiration		Respiration after addition of glucose	
	μl O ₂	μl CO ₂	μl O ₂	μl CO ₂
0—2	1,184	968	1,533	1,422
	1,277	1,023	1,582	1,411
	1,395	1,238	1,581	1,486
2—4	780	715	978	986
	798	754	939	958
	778	723	944	925
4—7	524	519	639	646
	589	600	595	636
7—10	517	517	582	611
	534	550	493	521
10—20	391	372	442	468
	322	283	413	408
	366	344	400	402
20—30	301	253	363	344
	234	185	311	284
	276	240	359	350

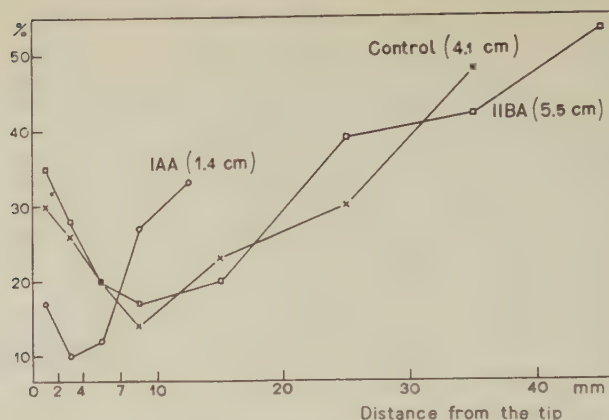


Figure 3. The increase in the O_2 uptake caused by glucose supply in per cent of the endogenous O_2 uptake. The endogenous O_2 uptake was measured during the period 3—6 hours after the excision. Then glucose was added to a concentration of 0.05 M and the respiration determined for another 2 hours. The figures in parentheses show the growth in length of the roots during the culture period.

The first problem — that of the rate of respiration in different parts of the root — has been dealt with in the paper by Eliasson. It is chiefly a problem of units of respiring matter (cf. James). Mainly for the purpose of giving a picture of the experimental data from which the other values are calculated, the values of the oxygen uptake and the carbon dioxide output per unit fresh weight for normal roots are given in table 1. There is a strong decrease particularly during the extension growth. Wanner has obtained similar values with the same material.

In determinations of R.Q.s and respiratory drifts, however, the only requirement on the unit of respiring material is that it must be as good a measure as possible of the respiratory capacity of parallel samples. As such a measure the fresh weight is quite satisfactory. Also when studying how the effect of glucose on the respiration varies in different parts of the root, it is possible to avoid the difficulties regarding the unit of respiring matter by comparing the increase in respiration caused by the addition of glucose calculated as a percentage. In figure 3 the percentual increase in oxygen uptake for different parts of the roots investigated is recorded. The effect of the glucose is considerably less in the final part of the extension zone than in the other parts. This may be considered in connexion with the fact that the decrease of the oxygen uptake without external substrate is smallest in the same part of the root (figure 1). The results imply that the respiration in the latter part of the extension zone and in the beginning of the differentia-

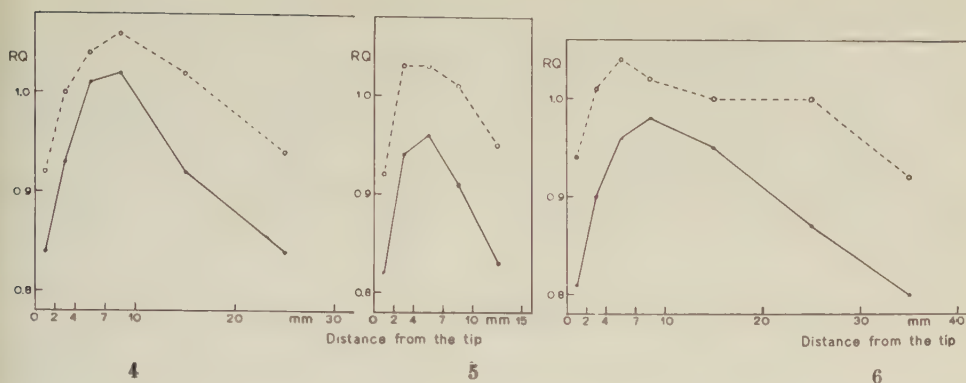


Figure 4—6. *The variation of the R.Q. with the distance from the tip.* Solid lines: Without substrate, during the period 3—6 hours after the excision. Broken lines: After addition of glucose, 6.5—8.5 hours after excision. The seedlings cultured for two days in sterile growth solution at $22^{\circ} C$ in the dark. Figure 4: without growth substances, growth in length 4.1 cm. Figure 5: IAA, $2 \times 10^{-8} M$, growth in length 2.1 cm. Figure 6: IIBA $3 \times 10^{-9} M$, growth in length 5.3 cm. — The points on the curves represent mean values of two or three experimental figures.

tion zone is not so easily affected by lack of substrate as it is in meristematic tissue and in older parts of the root.

The above suggestion is supported by the fact that the R.Q. shows a corresponding variation with the distance from the tip (figures 4—6). Both with and without supplied substrate the R.Q. shows a maximum at the end of the extension zone and is much lower in the apical and the basal parts of the root. This suggests that the respiratory material in this part of the root even in endogenous respiration is mainly carbohydrates. If this is true, it will explain why the need for substrate supply is not so great for this part of the root. Noteworthy is also the fact, that in this part the increase of R.Q. as a consequence of the glucose supply is considerably less than in the meristem and in the mature tissue. Further, a comparison with the experiments in which the segments were kept in the glucose solution for the entire period (figure 2) shows that the R.Q. in these parts of the root will only be partly restored as a consequence of the glucose supply.

From figures 3—6 it is apparent that the differences between roots with different growth rates are mainly a consequence of the fact that the length of the extension zone is changed with the growth rate. Thus these differences may be interpreted as resulting from changes in the growth and the morphology of the roots. In other respects the variation with the distance from

the tip is, regarding both the effect of the glucose and the R.Q., of a similar nature for roots grown in IAA or IIBA as for control roots. The fact that the maximal R.Q. values, particularly in endogenous respiration, are somewhat lower for IAA and IIBA roots suggests that the protoplasmic constituents in these roots are broken down more easily than in normal roots. The difference, however, is not fully significant. On the whole the results coincide with the earlier published evidence that there is no direct effect of the growth substances on the metabolism of the root cells.

Discussion

It seems reasonable to assume that the respiratory substrate in roots attached to the plant is mainly carbohydrates, which are transported there from the endosperm (cf. the results of Albaum and Eichel regarding the metabolism of oat seedlings during germination). After the excision the carbohydrates available for the respiratory processes are rapidly exhausted with a decrease of the respiration as a consequence. Simultaneously there is a decrease in the R.Q. Kandler (1950) has obtained a similar decrease of the R.Q. in excised roots of *Zea*. This decrease was counteracted by supply of glucose to the roots in the same way as in the experiments described here. On the other hand, Baldovinos for *Zea* roots and Machlis for barley roots obtained an increase of the R.Q. with the time after the excision.

The observed decrease of the R.Q. may according to the facts earlier discussed be due to: 1. the cessation of reduction processes involved in synthesis of cell substances caused by lack of substrate and respiratory energy, 2. utilization of reduced protoplasmic substances in the respiration. Kandler (1950), dealing with the theory of Ruhland et al. about an «aerobic fermentation» in embryonic tissue, considered the possibility that the increase of the R.Q. in such tissue may be due to fermentation processes induced by lack of oxygen. As a third possible cause of the decrease of the R.Q. the inhibition, due to lack of substrate, of such hypothetical fermentation processes may thus be considered.

As long as there is no accumulation of reduced degradation products like ethyl alcohol or lactic acid it seems, however, inappropriate to speak about fermentation. If the reduced substances are used for formation of normal cell constituents, the processes, from a physiological point of view, are of quite another nature than fermentation, an important characteristic of which is accumulation of degradation products. The fact that the carbon dioxide output observed in the absence of molecular oxygen in higher plants is not always followed by a corresponding accumulation of such products (see Stiles and Leach) indicates that the phenomenon usually classified as «anaerobic respiration» is not necessarily the same as fermentation (cf. also Goddard).

The increase of the R.Q. above the theoretical value caused by the reduction processes connected with the biosynthesis of new cell substances is relatively moderate even in parts of the root where there is an intense new formation of protoplasm. According to the material presented in this paper the increase for the growth zones of wheat roots will be from 1.0 to 1.1—1.2. Tamiya obtained for *Aspergillus* an increase of the same magnitude above the theoretically calculated combustion quotient of the substrate. As a nitrogen source he used ammonium salts. Among others Yamagata has shown that the increase becomes considerably greater if the nitrogen is supplied as nitrate. In this case he obtained R.Q. values about 1.5 when using sucrose or mannose as the substrate. These high values were connected with a high growth rate. Kandler (1950) obtained R.Q. values up to 1.3 for excised *Zea* roots on a glucose-nitrate medium.

Both the R.Q. gradient (figures 4—6) and the gradient of the »glucose effect» (figure 3) indicate that the available amount of substrate of the carbohydrate type is greater in the part of the root where the extension growth has just been completed than in other parts. It is, perhaps, not the substrate in the cytoplasm at the excision, but that transported there from the vacuole or from the cell wall which is the more important. The high sensibility to lack of substrate showed by the meristematic tissue thus may be due to the fact that the proportion between the cytoplasm on the one hand and vacuoles and cell walls which may serve as substrate reservoirs on the other is quite another than in elongated cells. Besides, the fact that the cytoplasmic surfaces in embryonic cells are small in relation to the amount of cytoplasm must have an unfavourable effect on the absorbing capacity of the cytoplasm.

During the extension growth the most apparent changes of the cells are the increase in size of the vacuole and the increase of the cytoplasmic surfaces. Probably this is connected with important changes of the cytoplasm (cf. Morgan and Reith). After the cessation of the cell elongation there is a gradual change in the cytoplasm, among other things resulting in a decrease in the respiration per unit nitrogen (see Eliasson). Changes of this nature may affect the content of carbohydrates available for respiration in these parts of the root. At the end of the extension zone the plasma membranes may be supposed to be in a labile state. This means, on the one hand, that the organic substances dissolved in the cell sap may be easily absorbed by the cytoplasm and used as respiratory material, on the other hand, that there may be a dissolution of the substances deposited in the cell wall. Each of these possibilities may explain the results obtained. An equilibrium between carbohydrates in the cytoplasm and in the cell wall seems, however, to be rather probable in this part of the root where deposition of new cell wall material occurs.

There is considerable evidence of a close contact between the cytoplasm and the cell wall during growth (cf. Frey-Wyssling 1950). The processes in the wall are probably regulated by enzyme molecules in direct contact with the wall or even penetrating the intermicellar spaces thereof. Bryan and Newcomb found a high pectin methylesterase activity in the wall fraction obtained from growing cells of tobacco pith. The changes that occur in primary walls are reversible and chemical substances may be removed or replaced by others (Esau, p. 38). Frey-Wyssling (1952) has given electron-microscopic evidence of a dissolution of cellulose microfibrils close to the site where other fibrils are synthesized. Even if such a dissolution of cellulose fibrils is not a general feature of normal wall growth (cf. the mechanism of wall growth proposed by Roelofsen and Houwink), it is probable that the growth is dependent of a continuously occurring breakdown of chemical bonds in the intermicellar substances and the formation of new ones (cf. Kerr). Thus lack of soluble carbohydrates should lead to predominance of the dissolvent processes over the deposition of new cell wall material. This coincides with the observation by Brown and Sutcliffe that the cellulose content of excised sections from the extension zone of *Zea* roots decreased if the sections were kept without sugar supply.

Thus there is strong evidence that the conditions observed regarding R.Q. and the »glucose effect» are due to an increased supply of soluble carbohydrates to the cytoplasm in the region of the root where the extension growth ends. This is probably due to the fact that the synthesis of cell wall substances is to a certain degree reversible in this region, whereas the wall becomes protected to dissolving enzymes when the cells get older.

Summary

The respiration of serial segments of wheat roots has been determined. There is a strong decrease in respiration and in R.Q. during the first hours after the excision. This decrease is counteracted by glucose supply.

The decrease of the R.Q. is greater in the meristem and in the mature tissue than in a region consisting of the last part of the extension zone and the first part of the differentiation zone. Besides, in this region the effect of the glucose is less than on other parts of the root. These facts can be explained by the assumption that the deposition of cell wall material is to a certain degree reversible and that, in the absence of substrate, there occurs a dissolution of wall substances.

Roots, whose growth has been inhibited or stimulated by growth substances, exhibit the same features as normal roots in the respects investigated.

References

- Albaum, H. G. & Eichel, B.: The relationship between growth and metabolism in the oat seedling. — *Amer. J. Bot.* 30: 18, 1943.

- Baldovinos, G.: Reacciones respiratorias de los sistemas meristematicos de la raiz primaria de maiz. — *Rev. Soc. Mex. Hist. Nat.* 11: 77. 1950.
- Brown, R. & Sutcliffe, J. F.: The effects of sugar and potassium on extension growth in the root. — *J. Exp. Bot.* 1: 88. 1950.
- Bryan, W. H. & Newcomb, E. H.: Stimulation of pectin methylesterase activity of cultured tobacco pith by indoleacetic acid. — *Physiol. Plant.* 7: 290. 1954.
- Eliasson, L.: The connection between the respiratory gradient and the growth rate in wheat roots. — *Physiol. Plant.* 8: 374. 1955.
- Esau, K.: *Plant Anatomy*. — New-York 1953.
- Frey-Wyssling, A.: Physiology of cell wall growth. — *Ann. Rev. Plant Physiol.* 1: 169. 1950.
- Growth of plant cell walls. — *Symp. Soc. Exper. Biol.* 6: 320. 1952.
- Goddard, D. R.: Anaerobic respiration or fermentation. — *Science*. 101: 352. 1945.
- James, W. O.: *Plant Respiration*. — Oxford 1953.
- Kandler, O.: Untersuchungen über den Zusammenhang zwischen Atmungsstoffwechsel und Wachstumsvorgängen bei in vitro kultivierten Maiswurzeln. — *Z. Naturforschung* 5 b: 203. 1950.
- Über den „Synthetischen Wirkungsgrad“ in vitro kultivierten Embryonen, Wurzeln und Sprosse. — *Z. Naturforschung* 8 b: 109. 1953.
- Kerr, T.: Growth and structure of the primary wall. — *Plant Growth Substances*. Univ. Wisconsin Press, Madison 1951.
- Machlis, L.: The respiratory gradient in barley roots. — *Amer. J. Bot.* 31: 281. 1944.
- Morgan, C. & Reith, W. S.: The compositions and quantitative relations of protein and related fractions in developing root cells. — *J. Exp. Bot.* 5: 119. 1954.
- Roelofsen, P. A. & Houwink, A. L.: Architecture and growth of the primary cell wall in some plant hairs and in the *Phycomyces* sporangiophore. — *Acta Bot. Neerl.* 2: 218. 1953.
- Ruhland, W. & Ramshorn, K.: Aërobe Gärung in aktiven pflanzlichen Meristemen. — *Planta* 28: 31. 1938.
- Stiles, W. & Leach, W.: *Respiration in plants*. — Third Ed. London 1952.
- Tamiya, H.: Zur Theorie des respiratorischen Quotienten nebst einer Bemerkung über den Einfluss der oxydoreduktiven Zellvorgänge auf den Gaswechsel der Zellen. — *Acta Phytoch.* 6: 227. 1932.
- Umbreit, W. W., Burris, R. H. & Stauffer, J. F.: *Manometric techniques and tissue metabolism*. — Burgess Publ. Co., Minneapolis 1949.
- Wanner, H.: The zonal graduation of respiratory intensity in the root. — *Ark. Bot.* 31 A: 9. 1944.
- Yamagata, S.: Über den Einfluss der Stickstoffquelle auf den Gaswechsel des Schimmelpilzes. Beiträge zur Physiologie der Nitrataassimilation. I. — *Acta Phytoch.* 8: 117. 1934.

The Stomata as a Hydrophotic Regulator of the Water Deficit of the Plant

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Earlier investigations (Stålfelt 1929, 1932) have shown that three kinds of movements can be distinguished in the guard cells of the stomata:

1. *Photoactive movements*, *i.e.*, those occurring as a result of processes within the guard cells (the guard cells being »active»), and which are initiated and driven by light energy. In those cases in which the guard-cell movement is purely photic and is thus uninfluenced by other processes, it follows the product law. This implies that the aperture is increased by the quantity of light absorbed. As a rule, however, the guard-cell movement is not purely photic, but is dependent on other factors as well, particularly the water deficit, *i.e.*, the quantity of water utilized for saturation of the leaf. The quantity of light required to produce an opening movement of a certain size is usually dependent on the water deficit of the leaf. With a certain deficit — the *optimal* — the light requirement is minimal; the photoactive opening movement then takes place undisturbed by other processes. With both a higher and a lower deficit there are, on the contrary, additional reactions which cause closing movements; they therefore counteract the photoactive opening movement, so that it requires more light.

2. *Passive opening and closing movements*. With a *suboptimal* water deficit, the guard cells are pressed together by the epidermal cells. When the deficit increases, the pressure is decreased and the guard cells open, and *vice versa*. The movements are thus determined by forces outside the guard cells; the cells themselves react only *passively*. That it is a question of a disturbance

in the equilibrium between the mutual pressure exerted by the guard cells and by the epidermal cells surrounding them is shown by the following observations, among others. The guard cells close if they are punctured by a micromanipulator (Heath 1938) and open if the turgor of the epidermal cells is decreased by, for example, trauma (Linsbauer 1917, Monzi 1938), transpiration (Stålfelt 1929) or osmotic suction (Monzi 1938, Mouravieff 1951). Opening nevertheless requires the guard cells to be in the «expansion phase» («Spannungsphase»), i.e., for their turgor pressure to be positive.

3. *Hydroactive movements*. When the water deficit has exceeded a certain threshold value — the borderline of the *supraoptimal* area — a closing movement is produced; it is initially weak, but with a rising water deficit it increases rapidly in strength, so that the stomata are closed even in light. This movement has been denoted as the *hydroactive* closing movement because it is initiated by the water deficit, and occurs owing to processes within the actual guard cells. (In their survey, Yemm and Willis (1954, p. 393) have happened to give the term «hydroactive» movement a meaning that differs partly from the original meaning.)

The photoactive opening movement is inhibited or made to regress by the hydroactive closing movement. A variable equilibrium is present between the former and the latter movement; it is altered automatically with the deficit, and it regulates the stomatal width at such values that the deficit is kept within certain limits. The two processes together constitute a *hydrophotic regulator* for the water consumption of the plant (Stålfelt 1929, 1932).

The effect of the hydroactive closing movement is maximal at a deficit which is a few per cent higher than the deficit (threshold value) initiating the movement. At a light intensity of 8000 M.C., the closing movement starts in *e.g.* *Vicia Faba* at a deficit of 3 per cent, but is completed only at a deficit of 5 per cent (Stålfelt 1929). Other threshold values are: *Betula*, 4 per cent (Stålfelt 1932); *Cissys sicyoides*, 3—4 per cent (Thomas 1949) and *Chrysanthemum maximum*, 5 per cent of the water content of the fully turgid leaf (presumably corresponding to 3—4 per cent of the fresh weight of the turgescient leaf) (Yemm and Willis 1954). Pisek and Berger (1938) found that, under natural conditions, closure took place in the majority of herbs at a deficit between 10 and 15 per cent. Such cases have subsequently been thoroughly analyzed by Pisek and Winkler (1953). Their work shows that the threshold value of the water deficit — measured at 300, 2000 and 10,000 M.C. — changes with the age and exposure of the leaf. Shade forms are more sensitive than sun forms, *e.g.* the threshold value of shade needles of *Picea excelsa* was found to be 5—6 per cent, whereas that of the sun needles was 8—9 per cent. The value also varies with the ecological features and with the species properties. The threshold value for

the deficit was found to be 2—2.5 per cent in *Fagus*, 3—4 in *Oxalis*, 4—6 in *Asarum*, 5 in *Quercus*, 6—8 in *Stachys* and 9—10 per cent in summer-green, sun-exposed herbs in general.

The higher the intensity of light, the greater is the deficit required to induce a complete closing movement (Stålfelt 1929, van den Honert 1941, Pisek and Winkler 1953).

Further analysis of the stomatal mechanism presupposes, among other matters, knowledge of the changes in the osmotic value and of their relation to the movements of the guard cells and to the water deficit.

As early as 1914, Iljin showed that the osmotic value changes with the movements of the guard cells. The value was up to 70 or 80 atmospheres higher in open guard cells than in the surrounding epidermal cells, but fell during stomatal closure, irrespective of whether it was induced by darkness or by great water loss. These results were confirmed by Steinberger (1922), who demonstrated further that the relation between the guard-cell movements and the osmotic value also appeared in saccharophylla *Allium* species; consequently, it is not invariably related to starch metabolism.

Wilting leaves were used by Iljin and Steinberger for a study of the effect of the water deficit. It can, however, be presumed that the osmotic value is influenced even by a smaller deficit than that causing wilting. The following questions therefore arise. At what deficit does the osmotic value of the guard cells start to fall? Does the fall in the osmotic value occur before, concurrently with or after the initial phase of stomatal closure? The present investigation was made chiefly with a view to investigating these relations.

Methods

Solutions of saccharose were used for measurement of the *osmotic value*. The glass slide, which had a spherical groove in the centre, also served as a plasmolysis vessel. The sections could therefore be examined directly without transfer to another slide, although it proved necessary in some cases to move the sections from the groove onto the plane surface of the slide. Two sections and 40 pairs of guard cells were examined in each specimen. The solution causing an approximately 50 per cent incidence of plasmolysis was taken as the incipient plasmolytic value. The determinations were made when the sections had remained in the solution for 30—45 minutes. Plasmolysis had by then attained a maximum, and subsequently regressed. The fact that plasmolysis of the guard cells does not reach any more stable state, but regresses rapidly was already observed by Steinberger (1922) and by Iljin (1923).

The specimens were taken from the central parts of the leaves, but not from the apical, basal or marginal areas.

Measurement of the width of the stomatal aperture was performed with the immersion method (Stålfelt 1929, pp. 188—190). A piece of leaf is excised and placed

in paraffin under an immersion objective (without cover-glass); 20 stomata are then measured with an ocular micrometer.

The *water deficit* of the leaf may undergo rapid changes. It must therefore be measured with a fast-weighing scales. A Sartorius selecta was used for this purpose.

Some difficulty is caused by the fact that the term deficit is ill-defined. As a rule, the water deficit of a leaf denotes the quantity of water that must be taken up by it to reach saturation. The saturation value is not, however, constant but varies with time. In the present investigation, the deficit has been calculated as the difference between the weight of the leaf on measurement and its weight when it had stood in water, in a closed vessel and in darkness for 12—16 hours.

The same leaf was used for several determinations in the course of a day. In order to maintain the proportion between the venous network and the parenchyma, the specimens were taken in such a way that not only the flatter parts of the blade were excised, but also the corresponding parts of the midrib.

The *experimental object* consisted of leaves of *Vicia Faba*, *Rumex acetosa* and *Rheum rhaponticum*. The leaves were severed, placed in darkness and saturated moisture, and were not used for experiments until the following day. In leaves treated in this way, the osmotic value was the same in the guard cells and in the epidermal cells, provided that the leaves had not suffered from any great lack of water during the immediately preceding days.

The *source of light* consisted of 1000-watt lamps with a plane glow body. The light was cooled with running water, of which the depth could be regulated.

The *experimental temperature* was kept at 20—22° C. The specimen leaf was placed in a glass dish with water-saturated filter paper; the dish was then placed in the light adapted for the experiment. On *removal of the specimen*, the leaf was weighed, and a piece of the blade was excised. The leaf was then re-weighed and was immediately returned to the light. Part of the specimen was used for measurement of the width of the stomata, and the remainder for plasmolysis. Further specimens were removed at fixed intervals.

Experimental

1. *Objects with a sub optimal water deficit*

The water deficit of a severed leaf can be regulated within certain limits by modifying the water supply and transpiration. Considerable difficulties are, on the contrary, encountered in keeping the water deficit constant or practically constant, *e.g.* between 1 and 3 per cent, in an illuminated leaf. This is because the leaf transpires even in the moisture-saturated chamber; moreover, the water suction is altered owing to the cut surface. With the intensities of light used — as a rule 7200 M.C. — the change usually consisted of an increase in the deficit. Sometimes, however, the deficit remained fairly constant and in other cases it decreased, *i.e.*, the turgor increased in light.

Figure 1 shows one of the experiments in which the water deficit remained within the ± 2 per cent limit. The decrease in the deficit during the subsequent

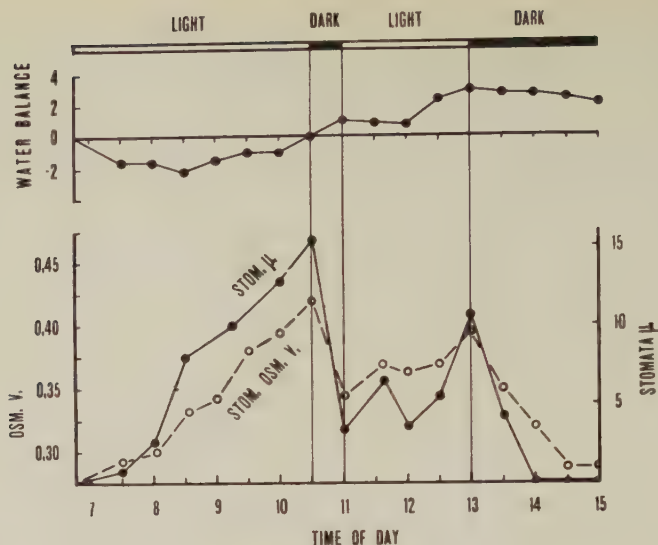


Figure 1. Relation of the width of the stomatal aperture to the osmotic value and to a suboptimal water deficit. The leaves, which had stood in darkness and saturated moisture for 16 hours, were exposed to 7200 M.C. from 6,45–10,30 and from 11,00–13,00, and were in darkness from 10,30–11,00. — Water balance=increase or decrease, respectively, in water content of the leaf, expressed in % of fresh weight of the leaf. (A negative value is identical with a positive deficit.) — The deficit first increased to 2 % and then decreased to -2.5 %. It was thus *suboptimal* during the whole experiment. Stomata μ and Stom. μ =width of aperture in μ (mean of 20 values). Osm. v.=incipient plasmolysis, i.e., the molar value of the cane sugar solution at which half of the guard cells (40 pairs examined) in the specimen showed some degree of plasmolysis. — *Vicia Faba*.

part of the experiment is a result of the change from light to darkness. The determinations were made at approximately half-hour intervals between 6,45 and 15,00 hrs. On two occasions, darkness was substituted for light. During the second dark period (13,00–15,00), the water supply to the leaf was interrupted, in order to prevent a further rise in turgor.

Throughout the experiment, the deficit was *suboptimal*. The movement followed the osmotic value of the cells in both light and darkness. In light, the rise was relatively slow — the light intensity was only 7200 M.C. — whereas the decrease in darkness occurred more rapidly. As demonstrated earlier (Stålfelt 1927, p. 248), the osmotic value increased with the quantity of light.

The course is in agreement with the traditional conception of the relation between the width of the stomata and light, i.e., the stomata open in light and close in darkness, the cause being the changes in the osmotic value due to the

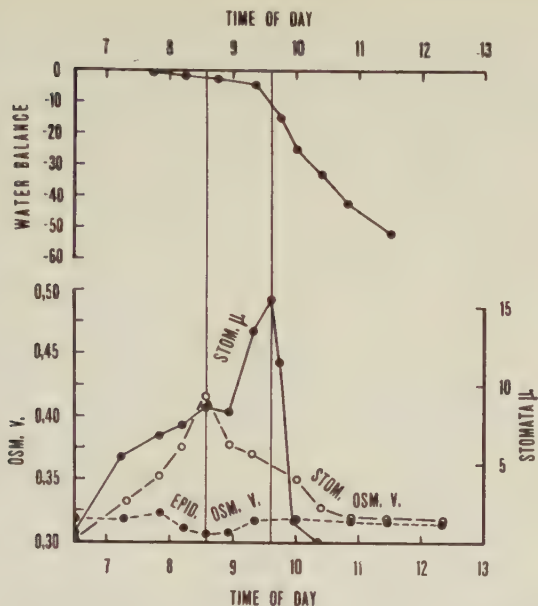
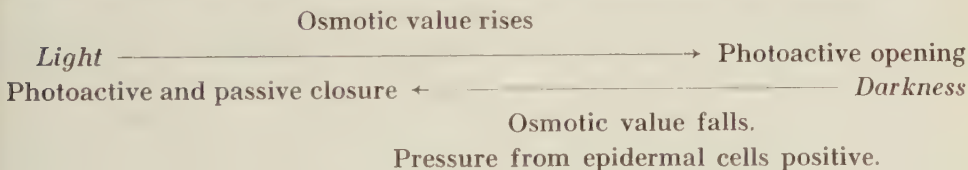


Figure 2. Same kind of experiment as in Figure 1, except that the object was illuminated throughout the experiment. Light intensity 7200 M.C. The water supply was interrupted after 3 hours to hasten the increase in the deficit. — The deficit initiates regression of the osmotic value of the guard cells but not of the epidermal cells. At about the same time, the deficit reaches and exceeds the optimal value, i.e., the range of water loss (approx. 3 %) in which the pressure of the epidermal cells on the guard cells is minimal. In this area, the increase in the aperture is *passive*. A somewhat higher deficit — the *supraoptimal* — initiates a rapid closing movement: *hydroactive closure*. — Otherwise as in Figure 1. — *Vicia Faba*.

effect of light. A particularly fine illustration of this relationship is found in a recent publication by Yemm and Willis (1954, p. 383).

Thus, with a *suboptimal water deficit*, the relation of the guard-cell movements to light and to the osmotic value can be depicted as follows:



2. Objects with *supraoptimal* and *optimal* deficits

Figure 2 shows the results of an experimental series performed in the same way as that stated in Figure 1. The only difference is that the object

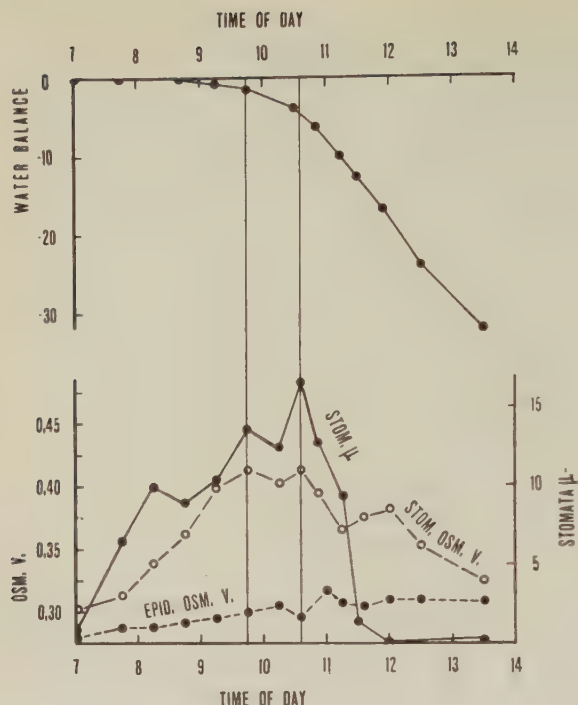


Figure 3. Same kind of experiment as in Figure 1, except that the object was illuminated throughout the experiment. Light intensity 7200 M.C. Water supply interrupted at 9,45, when the deficit was 2 %. — The osmotic value of the guard cells starts to decrease at a deficit of 2—5 %; somewhat later, or at a deficit of 5 %, hydroactive closure starts. — Otherwise as in Figure 1. — *Vicia Faba*.

was exposed to light throughout the experiment, and the water deficit rose to higher values. Initially, there was only a slow increase in the deficit. In order to hasten its development, the leaf was deprived of water at 9,40 hrs.

During the first two hours of illumination, the stomatal value followed the osmotic value of the guard cells, and both increased. However, when the deficit had reached the 2—4 per cent level, the osmotic value turned and it decreased, whereas a rapid increase took place in the width of the stomata, as a result of the decrease in pressure by the epidermal cells. Thus, this phase of opening was not only photoactive, but *passive* as well. When the deficit had increased further, and was between 4 and 15 per cent, the stomata started to close. Closure took place relatively quickly. It was not photoactive, there being no change in the light intensity, but was *hydroactive*, since it was initiated by the deficit.

Experiments of the same kind as that in Figure 2 are recorded in Figures 3 and 4. The experiment shown in Figure 4 was, however, made with leaves which, during the preceding days, had been exposed to water deficiency and, periodically, to wilting as well. This presumably explains the difference in the reaction with respect to the water content, which increased when the leaves were illuminated. It also explains why, at the beginning of the experi-

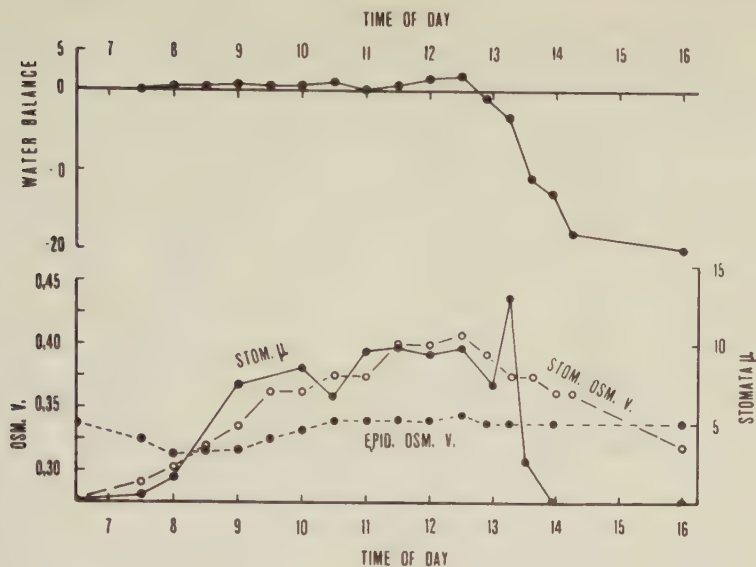


Figure 4. Same kind of experiment as in Figure 2, except for different pre-treatment. For a few days before the experiment, the leaf was subjected to water deficiency, which sometimes caused wilting. — At the beginning of the experiment (6,30) the leaf was turgid, but the turgor increased slightly until, after 6 hours (12,30), the leaf was deprived of water. The osmotic value falls with the water content of the leaf. A *passive opening movement* occurs at a deficit of 2—4 %. *Hydroactive closure* is initiated at a deficit of 4 %. — Otherwise as in Figure 1. — *Vicia Faba*.

ment (6,30 hrs), the osmotic value of the guard cells was lower than that of the epidermal cells. This phenomenon was also observed by Steinberger (1922) in experiments with leaves that had been exposed to wilting. Finally, it explains the fact that the walls of the guard cells bulged inwards and were lacking in turgor when the experiment was started. Despite their low osmotic value, the guard cells were able to take up water from the epidermal cells and to open. This is because the pressure in their walls is slight or non-existent, in contrast to that of the epidermal cells, which are distended and highly turgid.

The osmotic value of the epidermal cells exhibited, in distinction to that of the guard cells, only slight and irregular variations in experiments 2—4.

In experiment 5 (Figure 5), leaves of *Rheum rhaponticum* were used. A piece of the blade was excised and placed in darkness and saturated moisture for 16 hours, after which it was examined. As in the previous case, the water deficit initiated a decrease in both osmotic value and stomatal width. In these respects, *Rheum* did not differ from *Vicia Faba*.

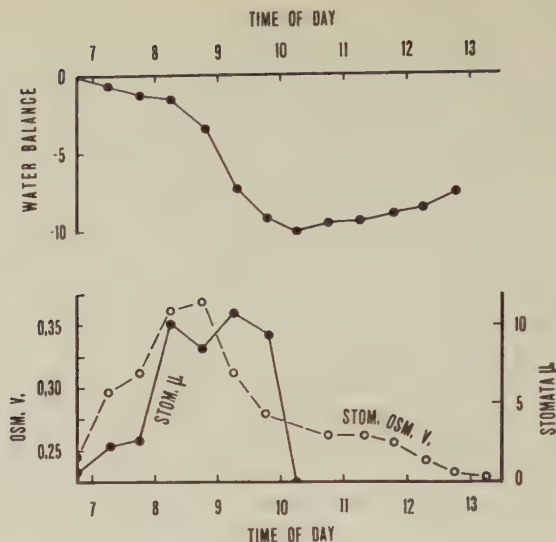


Figure 5. Same kind of experiment as in Figure 1, except that the object was illuminated throughout the experiment. Light intensity 7200 M.C. The water supply was not interrupted. The object was thus exposed to constant illumination and supply of water. — The water content of the leaf nevertheless falls from the start (owing to mucus formation on the cut surface) and continues to fall until closure of the stomata, after which a slight increase occurs. The osmotic value starts to fall at a deficit of 3–4 %. At a deficit of 6 %, hydroactive closure starts. — Otherwise as in Figure 1. — *Rheum rhaponticum*.

Work with *Rheum* nevertheless presented difficulties, since the cut surface was covered by mucus, which impeded the uptake of water and caused a rapid rise in the deficit. Consequently, *Rheum* was used in only a few experiments. Use was made instead of leaves of *Rumex acetosa* which, like the other experimental objects, was grown in the natural surroundings. The leaves of *Rumex* are larger than those of *Faba*, and the experiment can therefore proceed for a longer time before the blade has been used up by the removal of specimens. Furthermore, the changes in the deficit are more favourable, they take place more rapidly than in *Faba*, but more slowly than in *Rheum* and it is therefore easier to study their effects.

Experiment 6 (Figure 6) was performed with *Rumex acetosa*. The size of the leaf permitted the experiment to be prolonged for several hours and, owing to the slow rise in the deficit, the stomatal regulator had time to take effect and to limit the water loss. As in the previous experiment, the decrease in osmotic value and in stomatal width were initiated by even a slight water deficit (1–2 per cent). The hydroactive closing movement decreased the opening value so rapidly that the increase in the deficit ceased at an early

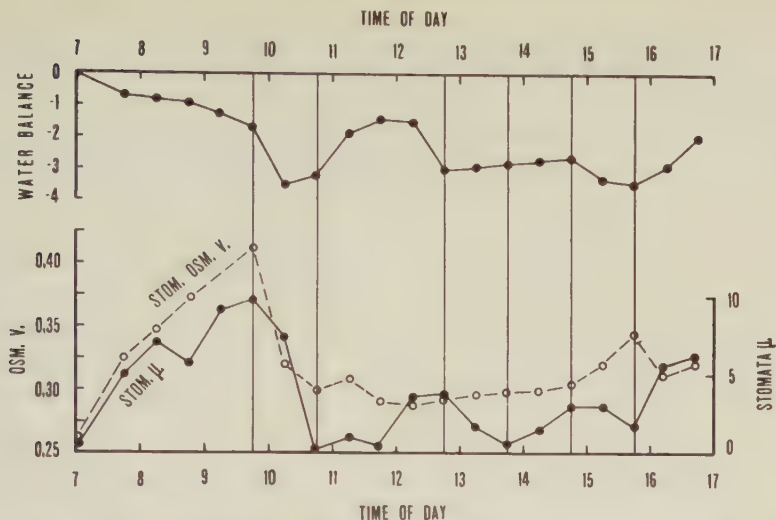


Figure 6. Same kind of experiment as in Figure 1, except that the object was illuminated throughout the experiment. Light intensity 7200 M.C. The water supply was not interrupted. — After exposure to light at 7.00 the osmotic value, stomatal width and deficit rise. When the deficit reaches 1—2 %, hydroactive closure occurs and the osmotic value starts to fall. When the stomata are almost closed, the water content of the leaf rises and the stomata open partly again; this increases the deficit and hydroactive closure once more takes place. The water deficit is regulated to values between 1.5 and 3.5 % by means of the variable equilibrium between photoactive opening and hydroactive closure. The experiment shows the action of the hydrophotic regulator, its initiation by the deficit and the regulation of the deficit by the regulator. — Otherwise as in Figure 1. — *Rumex acetosa*.

stage and the water balance of the leaf improved. The hydroactive closing reaction is weakened by the regression of the deficit; the photoactive opening reaction therefore once more dominates and a new opening phase starts. As a result, transpiration once again becomes greater, the deficit starts to be larger, the hydroactive closing reaction increases in strength for the second time, stomatal width decreases and development of the deficit ceases. By means of this balance between photoactive opening and hydroactive closure, the deficit is kept within relatively narrow limits.

In the previous experiment, this last phase of the hydrophotic regulating mechanism did not appear, since the deficit reached such a high level so rapidly that the water balance could not be restored during the experimental period.

Experiment 7 (Figure 7) is a repetition of experiment 6 and shows, on broad lines, the same course. After the stomatal width had fallen to 1—2 μ on account of hydroactive movements the water deficit slowly regressed.

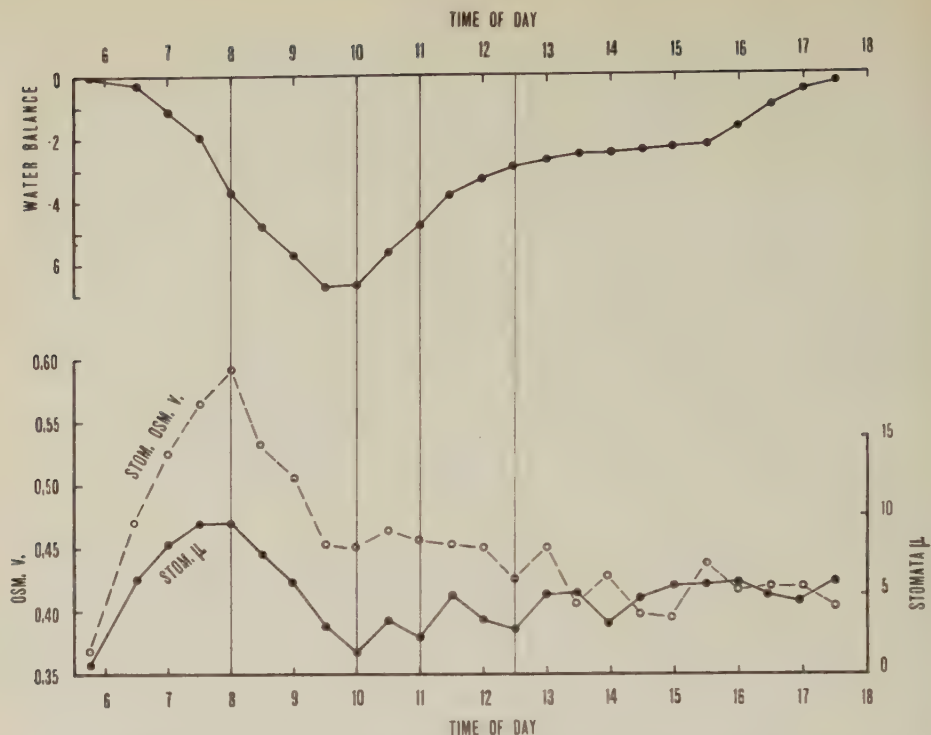


Figure 7. Repetition of the previous experiment, but the leaf was exposed to light with the lower side upward. The guard cells are therefore exposed to stronger light than in previous experiments, although the intensity is the same (7200 M.C.). — The rise in the osmotic value is greater than in preceding experiments, but stomatal opening ceases at the same value (about $10\ \mu$) and about the same deficit (2–4 %). When the stomatal aperture has decreased to $1\text{--}2\ \mu$, the water content starts to rise; hydroactive closure is thereby weakened, photoactive opening again starts to dominate and the stomata slowly open. — Otherwise as in Figure 1. — *Rumex acetosa*.

The hydroactive process was thereby weakened, and the stomata slowly reopened. During the secondary opening, the osmotic value fell. Consequently, the secondary opening is not a result of the changes in the osmotic values. Its cause is presumably to be sought in another process that is initiated by the deficit, and that is weakened with the regression of the water deficit.

The stomatal width could have been expected to return to $10\ \mu$ when the deficit decreased to 3–4 per cent, i.e., to the deficit value at which the primary opening reached a value of $10\ \mu$ for the aperture. This did not, however, occur. The experiment showed instead that the hydroactive closing reaction did not cease concurrently with the decrease in the deficit and its disappearance, but persisted as an *after-effect*. Because of this after-effect,

the opening cannot be as powerful as that occurring in the first instance, even though the external conditions are the same and the water balance of the leaf is also the same as on the primary opening. By means of this after-effect, the hydroactive reaction retains its controlling influence on the stomatal mechanism for some time after cessation of the deficit.

The Stomatal Effects of the Water Deficit

It is evident from the present experiments, as well as from those made earlier (Stålfelt 1929, 1932), that a causal relationship exists between the stomatal movements and the water deficit of the leaf, and that the deficit has several effects. Briefly, they can be described as follows.

With an *optimal* deficit, photoactive opening meets with inappreciable resistance or none at all: the epidermal cells exert little or no pressure on the guard cells; the hydroactive closure does not participate. The optimal deficit is confined to a narrow area immediately below the threshold value of the hydroactive reaction. In this area, the movement of the guard cells is determined by the light alone.

Thus, with an *optimal deficit*, the guard cells react according to the following schema:

Light —————→ Photoactive opening
 Photoactive closure ←———— Darkness

With a *supraoptimal* water deficit, the stomatal movements are, on the contrary, controlled by the *hydrophotic regulator*, i.e., by the variable equilibrium between photoactive opening and hydroactive closure. As already mentioned, the initiating deficit varies in different species. Exact determination of its magnitude requires frequent, consecutive measurements at close intervals (Stålfelt 1929). Such measurements could not be made in the present investigation. This was because it was necessary to use long time intervals, in order to save the leaf, so that the experiment could cover several hours. Consequently, in most cases, the specimens were taken only when the deficit had fallen far below the threshold value.

For this reason, the threshold values recorded in Table 1 are too high. According to an earlier analysis made on Faba, using short time intervals, the threshold value lies at about 3 per cent (Stålfelt 1929); in Table 1 it ranges from 1.5 to 10 per cent.

When the water deficit exceeds the threshold value to the supraoptimal area, the hydroactive closing reaction is elicited, transpiration decreases and the deficit regresses. The hydroactive closing reaction is thereby weakened.

the photoactive opening reaction once more dominates, the stomata are dilated, transpiration increases, the water deficit rises, the hydroactive closing reaction becomes stronger again, and so on. *The regulator thus acts in the same way as a relay. It causes an alternation between the opening and closing movements as long as the water deficit has not risen to those values at which the photoactive opening movement is entirely inhibited.*

The series of opening and closing movements should result in corresponding variations in transpiration. Continuous determinations of the transpiration have been made by Andersson, Hertz and Rufelt (1954). They found that the course of transpiration actually appears as a series of variations. It is apparent from their report that the variations take place within relatively narrow limits, and that the duration of the different periods is strikingly constant. Studies of similar variations have been made earlier by Boresch (1933), Gregory and Pearse (1937) and Went (1944). They are also reflected in the width of the stomata apparatus (Stålfelt 1927).

Thus, the water deficit initiates the following stomatal processes:

1. A *suboptimal deficit initiates passive opening and closing reactions* by means of a change in its value.

2. A *supraoptimal deficit initiates active closing reactions*. This phenomenon has been described earlier (Stålfelt 1929, Pisek and Winkler 1953).

3. The *osmotic value* of the guard cells *falls* when the deficit has reached certain values. In 25 out of 26 series in the present investigation, the osmotic value fell for this reason (Table 1); in one case it remained unchanged or continued to rise, although the deficit increased and stomatal closure took place.

Hydroactive closure is a time-consuming process; it takes some time before the effect of the initiating deficit becomes apparent as the onset of closure (see p. 573). Presumably, there is also some delay before the effect of the initiating deficit is manifest as a fall in the osmotic value of the guard cells. The investigation gives no information regarding the magnitude of these time intervals. As already mentioned, it was impossible on technical grounds to establish the time for these two phenomena with the same accuracy as on an earlier occasion, when the study was confined to the closure of the stomata cells (Stålfelt 1929). Consequently, it was also impossible to determine whether the decrease in the osmotic value took place before, concurrently with or after the onset of the closing movement. One fact nevertheless seems certain, *i.e.*, both the decrease in the osmotic value and the stomatal closure are initiated by the water deficit that lies close to the deficit values which are optimal for the photoactive opening movement. This implies that the optimal deficit is confined to a narrow segment of the water content

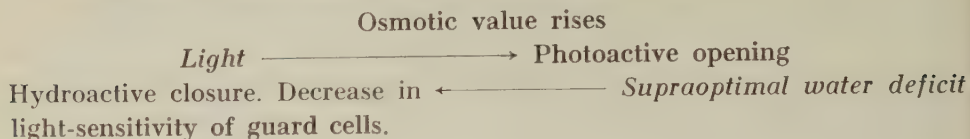
of the leaf, so that the hydroactive closing reaction takes effect as soon as the photoactive opening movement starts to reach high values.

4. A fourth kind of stomatal effect consists of the *decreased sensitivity of the guard cells to light* produced by the *supraoptimal* deficit. This may be inferred from the experimental series of such long duration that the deficit had not only had time to develop, but also to regress wholly or partly. Such an experiment is illustrated in Figure 7. It is seen that the photoactive opening reaction is more effective before the occurrence of the deficit maximum than after it. With a deficit of 2 per cent (at 7,20 hrs) *before* the deficit maximum, the stomatal aperture is 9–10 μ ; with the same deficit (at 15,45 hrs) *after* the maximum, the aperture is only 6 μ . During the later part of the experiment, when the deficit is in process of regressing, the photoactive opening reaction operates at the same deficit value (2–3 per cent) for 3 hours (12,30–15,30 hrs), but is then able to produce an aperture of only 2–7 μ . The large aperture values (9–10 μ) which were quickly reached before the deficit maximum no longer appear. The original sensitivity to light was restored only in leaves that had been water-saturated for several hours, as indicated by other experiments. *Thus, the supraoptimal deficit has an after-effect; it consists of a decrease for some time in the sensitivity of the guard cells to light.* A *safety mechanism* is inherent in this reaction; it protects the leaf from the rapidly developing supraoptimal water deficit which would occur during light, warm days, when the deficit — during its regression — reaches the optimal values for the photic opening process. If this safety mechanism did not exist, the guard cells would open rapidly when the deficit had fallen to the optimal level, and transpiration would increase rapidly again. Under natural conditions, the prerequisites for such variations are greatest during the early morning and forenoon, *i.e.*, with increasing light intensity and heat. The deficit would then increase in magnitude each time, and would expose the leaf to successively greater risks. For, the greater the transpiration, the greater will be the deficit before stomatal closure starts and has been completed.

In leaves exposed to large and rapidly developing deficits, the stomatal regulator is unable to start functioning in time, but the deficit reaches values which damage the mechanism. Under such conditions, the leaf withers without closure of the stomata (Stålfelt 1916, Iljin 1922, p. 672, Sayre 1926, Pisek and Winkler 1953, p. 271).

The photoactive opening mechanism is thus also controlled by a *sensitivity regulator*, which is initiated by the water deficit when it reaches supra-optimal degrees.

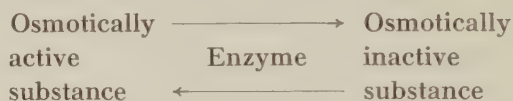
Consequently, the schema for the *hydropnotic regulator* is:



The Nature of the Stomatal Processes

The foregoing account has dealt only with the part played by the various stomatal processes in the opening and closing movements of the guard cells. It has not touched on their nature or on their interdependence.

a. The course of the *photoactive processes* with a suboptimal water deficit (Fig. 1, schema, p. 576) seems to be in agreement with the enzymic theory of the stomatal movements, as it has been formulated by, in particular, Iljin (1914, 1922, 1932) and Linsbauer (1927). Its essential principles can be presented schematically by the formula



Important additions to this theory have been made by Yin and Tung (1948), who found that the guard cells contain phosphorylase, and by Small and Crosbie-Baird (1942) and Williams and Barrett (1954), who established that the enzymic system of the guard cells acts so rapidly that distinct metabolic changes can be demonstrated already after 2—3 minutes.

Since the enzymic activity changes with the pH of the system (Hanes 1940), it is also dependent on the supply of respiratory oxygen (Linsbauer 1917) and on substances which change the pH, *i.e.*, both acids and alkalis (Nicolie 1925, Arends 1925) as well as salts (Iljin 1922, Schmetz 1925, and others). Because the pH changes are elicited by the alternations between light and darkness (Searth 1932, Pekarek 1933), the causal chain seems — at any rate in principle — to be closed (Sayre 1926, Searth 1932). Its main sequence would thus consist of the following links:



b. The *hydroactive processes* are of three kinds. The deficit initiates stomatal closure, decreases the osmotic value of the guard cells and lowers their sensitivity to light during the photoactive opening phase. The last-mentioned effect of the deficit may also be expressed as follows: hydroactive

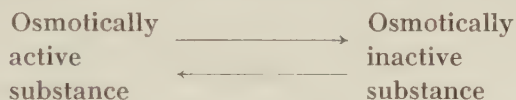
Table 1. *The size of the initiating water deficit (mean values).*

Species	Osmotic value of guard cells starts to fall at deficit %	Stomatal closure starts at deficit %	No. of experiments
<i>Vicia Faba</i>	3.6 (1—9)	6.7 (1.5—10)	14 (13)
<i>Rumex acetosa</i>	2.6 (1—5.2)	3.4 (1.8—5.2)	8
<i>Rheum rhaponticum</i>	1.5 (1—3.5)	4.4 (1—7.2)	4

closure has an after-effect which persists even after the deficit has regressed, and which makes the guard cells indisposed to photic opening.

Nothing is known with certainty about these reactions. Naturally, several possible explanations exist. Mention will be made of only a few of them.

By decreasing the *sensitivity to light* of the guard cells, the deficit impedes the photoactive opening process. The mechanism may consist of an action of the deficit on the enzymic reactions regulating the system



If this is the case, the deficit shifts the equilibrium to the right, or inhibits its path to the left.

The relation between *the deficit and hydroactive stomatal closure* seems to be complicated. The fact that the deficit influences the system of osmotic equilibrium is an argument in favour of the supposition that it does, in fact, initiate the hydroactive closing movement by means of this mechanism. The time difference between the onset of closure and the regression of the osmotic value (Table 1) also seems to point in this direction, since the former occurs later than the latter. In reality, the time difference nevertheless lacks value as proof. This is because it has not been established how long a period elapses between the moment when the deficit initiates the closing process and that when the process becomes apparent in the form of movements of the cells.

Moreover, it is found that in some of the present experiments, such as those recorded in Figures 6 and 7, there is a distinct synchronism between the osmotic value and the closing movement. To interpret this as an indication of the aforementioned relation nevertheless presupposes, as in the previous case, that the duration of the action of the deficit before the onset of closure is known.

Other observations point, however, in the opposite direction and suggest that hydroactive closure is not caused by the decrease in the osmotic value.

Firstly, there was one case in the present series in which closure occurred without a decrease in the osmotic value; in other cases closure was complete and took place rapidly, even though the decrease in the osmotic value was small (*e.g.* Figures 3 and 4). Secondly, it was found that closure proceeded rapidly, whereas the fall in the osmotic value was usually slow.

The question whether or not hydroactive closure is produced by a decrease in the osmotic value must therefore be left open at present.

Discussion

Among the factors influencing the movements of the stomata cells — light, pressure of the epidermal cells and the water deficit of the plant — the water deficit has the strongest and the most highly differentiated effect. It initiates closing movements, it causes cessation of photoactive opening, it produces changes in the sensitivity of the guard cells to light, it determines the turgor pressure and thereby the stomatal movements of passive nature.

At the borderline to the optimal area, the deficit passes rapidly from one mode of action to the other. Consequently, certain difficulties are encountered in following the different processes which then occur in succession. It is in particular the small breadth of the optimal range that hampers the analysis. A quantitatively insignificant change in the deficit suffices for one reaction to cease and another to start; an opening process may end and be succeeded by a closing process and *vice versa*. *As a result, the guard cells exhibit great sensitivity to changes in the environmental factors when the deficit is optimal.*

Even severing of a leaf may result in a change in the mode of reaction. Went (1944) observed that the stomata closed within a few minutes even when relatively little water was lost by transpiration. The cause may have been that the deficit was close to the threshold value for hydroactive closure, so that it rapidly exceeded this value when the water supply to the leaf was cut off. The same observation was made by Andersson, Hertz and Rufelt (1954). They found, however, that transpiration showed a transient rise before the fall which followed excision of the leaf; a transient increase will, in fact, result, if the first water loss elicits passive opening.

Went also found that no correlation existed between the width of the stomata and the suction force of the leaf, and that the stomata closed in the afternoon under conditions of constant humidity. The explanation is presumably that the deficit, and with it the stomatal width, can undergo changes without any alteration in the humidity of the air, and *vice versa*. Furthermore, the deficit can vary within wide limits without markedly influencing

the stomata if, during the immediately preceding hours, they have been exposed to hydroactive closure.

A change in the manner of reaction may also result from an increase in light intensity or from constant illumination, since transpiration is increased by light. If the water deficit is optimal, it will become supraoptimal in such cases, *i.e.*, increased light intensity or continued illumination then leads to stomatal closure, as was found by Nutman (1937).

If the deficit varies, the effects of light will also be variable. Earlier investigations have shown that the effects of light are, in fact, variable, as has been pointed out by Scarth and Shaw (1951).

Gregory *et al.* (1950, p. 2) have stated that in my earlier experiments (Stålfelt 1929, 1939) »no account was taken of a possible shock reaction of the stomata following excision of the sampled portion used for the measurement», and that »the number of stomata examined was always small». This criticism is correct in so far as the immersion method permits measurements of only a minor proportion of the stomata of a leaf. But, all methods for quantitative determinations of the stomatal width suffer from this limitation; this also applies to the porometer method used by Gregory *et al.* One of the reasons for which I did not choose the porometer method for my earlier investigations was that I wished to study the relation between the stomatal movements and the water deficit, and this method does not allow simultaneous and detailed measurements of the stomata and the deficit. The changes sometimes exhibited by the porometer values, and that have been interpreted as signs of shock reactions of the stomata, have actually no corresponding features in investigations with the immersion method. With an optimal water deficit, the guard cells react rapidly — both passively and hydroactively — to the changes in the deficit, but the causal relationship is naturally apparent only if the deficit is recorded simultaneously with the stomatal width. If, on the contrary, the deficit is not recorded, these hasty stomatal changes must seem to be inexplicable, and may give reason to put forward the »shock» hypothesis.

Gregory *et al.* (1950) have also studied the relation between the transpiration rate and the leaf water content; they concluded that »under the conditions of these experiments transpiration is quite independent of leaf water content» (p. 26). If this interpretation were correct, it would imply either that transpiration is independent of stomatal width, or that the latter is independent of the water deficit of the leaf. Both conclusions are, however, contradictory to the results of earlier experiments on these relations, and to those of the investigations reported in the foregoing. Nor could they be confirmed by Hygen (1953 b) who took up this matter for discussion. That Gregory *et al.* failed to reveal any quantitative relation between transpiration

and leaf water content is presumably to be ascribed to the fact — also pointed out by Hygen — that the experiments were designed in such a way that this relation was masked by other factors.

Gäumann and Jaag (1938, as well as earlier and later publications) and Gäumann (1942) made thorough investigations of the cuticular transpiration of plants. They based their measurements and calculations on the assumption that the stomata are open in light and closed in darkness, and that the opening movements are »unmittelbar von der Lichtmenge abhängig» (*e.g.* 1938, p. 58). Consequently, their method and conclusions rely on the supposition that a change in light produces a corresponding change in stomatal width (*e.g.* 1938, p. 62). Such a relation between the stomata and light nevertheless exists only with a suboptimal to optimal water deficit, and it is invariably brief and rapidly transient. In other cases it does not exist; in general, the guard cells do *not* follow the light. This relation was already known earlier. Under certain conditions, the stomata may partly open even at night, as has been demonstrated in numerous investigations (see bibliography: Stålfelt 1929, p. 334). An aperture of variable size is often found even in leaves that have stood in water, saturated humidity and darkness for several hours; this is also shown by the experiments recorded in Figures 3, 5, 6 and 7 in the present paper.

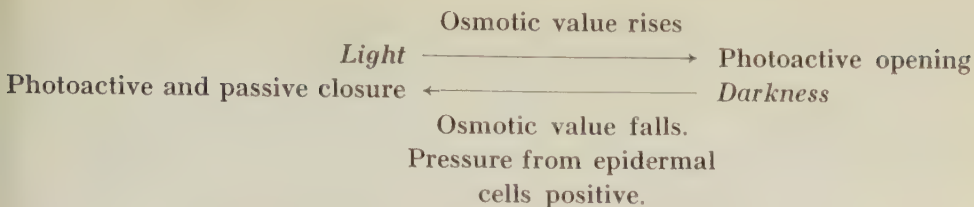
Judging by the data, Gäumann and Jaag used experimental objects whose stomata were partly open, and not entirely closed as they assumed. Hygen and Midgaard (1954), who reproduced these experiments, could not confirm their conclusion.

Summary

The object of the investigation was to study the relation between the movements of the guard cells and the osmotic value, as well as the influence of the water deficit of the leaf on these two phenomena.

The experimental objects consisted of leaves of *Vicia Faba*, *Rumex acetosa* and *Rheum rhaponticum*. The stomatal aperture, osmotic value and water deficit were measured at fixed intervals during the day. During this time, the leaves were kept in light and saturated humidity at a temperature of 20—22° C.

With a *suboptimal water deficit*, the guard cells are found to open in light and close in darkness. Their osmotic value rises during the opening movement and falls during the closing movement. The course is thus in agreement with the traditional conception of the stomatal movements as a function of the light and of the enzymic metabolism; it is represented by the schema:



With a *supraoptimal water deficit*, the movements of the guard cells are controlled mainly by the water deficit. Three kinds of effect can be distinguished:

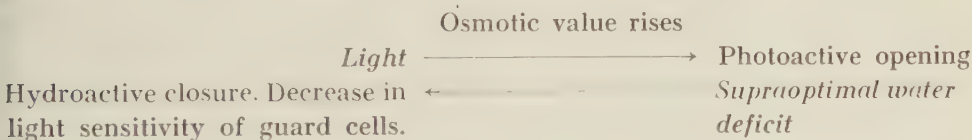
1. The deficit initiates stomatal closure. The process has been described earlier and denoted as the hydroactive stomatal closure (Stålfelt 1929). The hydroactive stomatal closure and the photoactive opening together form the *hydrophtic regulator* of the water balance.

2. The deficit decreases the osmotic value of the guard cells. It could not however, be determined whether the osmotic value starts to fall before, concurrently with or after initiation of the closing movements of the stomata.

3. The deficit decreases the light sensitivity of the guard cells to the photic opening process. This is because hydroactive closure has an after-effect, which persists for several hours after the deficit has decreased or regressed, and which makes the guard cells indisposed to photic opening. A *safety mechanism* is inherent in this inhibiting reaction; it protects the leaf from rapidly developing large water deficits which would arise on light, warm days, if the photoactive opening mechanism continued unchanged after the deficit had regressed owing to stomatal closure.

Thus, it is found that the photoactive opening reaction is also controlled by a *sensitivity regulator*, which is initiated by the deficit when it reaches high supraoptimal values.

If the safety mechanism is also included, the *hydrophtic regulator* can be represented by the schema:



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References

Andersson, N. E., Hertz, C. H. & Rufelt, H.: A new fast recording hygrometer for plant transpiration measurements. — *Physiol. Plant.* 7: 753. 1954.

- Arends, J.: Über den Einfluss chemischer Agenzien auf Stärkegehalt und osmotischen Wert der Spaltöffnungsschliesszellen. — *Planta* 1: 84. 1925.
- Boresch, K.: Zur graphischen Registrierung der Transpiration von Blättern. — *Planta* 20: 448. 1933.
- Gäumann, Ernst: Über die pflanzliche Transpiration. I. Die kutikulare Transpiration. — *Zeitschr. f. Bot.* 38: 225. 1942.
- & Jaag, O.: Untersuchungen über die stomatäre Transpiration. — *Ber. Schweiz. bot. Ges.* 48: 45. 1938.
- Gregory, F. G. & Pearse, H. L.: The effect on the behaviour of stomata of alternating periods of light and darkness of short duration. — *Ann. of Bot. N.S.* 1: 3, 1937.
- Miltorpe, F. L., Pearse, H. L. & Spencer, H. J.: Experimental studies of the factors controlling transpiration. I. Apparatus and experimental technique. — *Journ. Exptl. Bot.* 1 (1): 1. 1950 a.
- , —, —: Experimental studies of the factors controlling transpiration. II. The relations between transpiration rate and leaf water content. — *Journ. Exptl. Bot.* 1: 15. 1950 b.
- Hanes, C. S.: The reversible formation of starch from glucose-1-phosphate catalysed by potato phosphorylase. — *Proc. roy. Soc. London B* 129: 174. 1940.
- Heath, O. V. S.: An experimental investigation of the mechanism of stomatal movement. — *New Phytol.* 37: 385. 1938.
- van den Honert, T. H.: Experiments on the water household of tropical plants. I. Water balance in *Cissus sicyoides* L. — *Ann. of the Bot. Gardens, Buitenzorg.* 51: 58. 1941.
- Hygen, G.: On the transpiration decline in excised plants. — *Det Norske Vidensk. Akad. Skr. I. Mat. Naturv. Kl.* 1: 84. 1953 b.
- & Midgaard, E.: A reinvestigation of the influence of varying air humidity on cuticular transpiration in *Pinus silvestris* L. — *Physiol. Plant.* 7: 128. 1954.
- Iljin, W. S.: Die Regulierung der Spaltöffnungen im Zusammenhange mit der Veränderung des osmotischen Druckes. — *Beih. Bot. Centralbl.* 1: Abt. 32. 1914.
- Wirkung der Kationen von Salzen auf den Zerfall und die Bildung von Stärke in der Pflanze. — *Biochem. Z.* 132: 494. 1922 a.
- Die Wirkung hochkonzentrierter Lösung auf die Stärkebildung in den Spaltöffnungen der Pflanzen. — *Jb. wiss. Bot.* 61: 697. 1922 b.
- Über den Einfluss des Welkens der Pflanzen auf die Regulierung der Spaltöffnungen. *Jb. wiss. Bot.* 61: 670. 1922 c.
- Die Permeabilität des Plasmas für Salze und die Anatonose. — *Studies from the Plant Physiol. Lab. of Charles Univ. Prague* 1: 97. 1923.
- Über Öffnen der Stomata bei starkem Welken der Pflanzen. — *Jb. wiss. Bot.* 77: 220. 1932.
- Linsbauer, K.: Beiträge zur Kenntnis der Spaltöffnungsbewegungen. — *Flora* 109: 100. 1917.
- Weitere Beobachtungen an Spaltöffnungen. — *Planta* 3: 527. 1927.
- Monzi, M.: Beeinflussung der Spaltöffnungsweite durch plötzliches Wassersperren und -zuführen, mit besonderer Berücksichtigung der Spaltöffnungsbewegungen zur Regenzeit. — *Jap. Journ. Bot.* 9: 113. 1938.
- Die Mitwirkung der Stomata-Nebenzellen auf die Spaltöffnungsbewegung. — *Ebenda* 9: 373. 1939.
- Mouravieff, Igor: Action de l'hydratation des cellules épidermiques sur l'appareil stomatique. — *C. r. Acad. Sci.* 232: 1507. 1951.
- Nicolaič, M.: Beiträge zur Physiologie der Spaltöffnungsbewegung. — *Beih. bot. Zbl.* 41: 309. 1925.

- Nutman, F. J.: Studies of the physiology of *Coffea arabica*. — Ann. Bot. N. S. 1: 353. 1937.
- Pekarek, J.: Über die Aziditätsverhältnisse in den Epidermis- und Schliesszellen bei *Rumex acetosa* im Licht und im Dunkeln. — *Planta* 21: 419. 1933.
- Pisek, A. & Berger, E.: Kutikuläre Transpiration und Trockenresistenz isolierter Blätter und Sprosse. — *Planta* 28: 124. 1938.
- & Winkler, E.: Die Schliessbewegung der Stomata bei ökologisch verschiedenen Pflanzentypen in Abhängigkeit vom Wassersättigungszustand der Blätter und vom Licht. — *Planta* 42: 253. 1953.
- Sayre, J.: Physiology of Stomata of *Rumex patientia*. — Papers from the Dep. of Bot. The Ohio State Univ. 171: 233. 1926.
- Scarsh, G. W.: Mechanism of the action of light and other factors on stomatal movement. — *Plant Physiol.* 7: 481. 1932.
- & Shaw, Michael: Stomatal movement and photosynthesis in *Pelargonium*. I. Effects of light and carbon dioxide. — *Plant. Physiol.* 26: 207. 1951 a.
- Schmetz, L.: Untersuchungen über den Einfluss einiger Aussenfaktoren auf den Stärkebau im Laubblatt. — *Bot. Archiv.* 10: 16. 1925.
- Small, J. & Crosbie-Baird, J.: PH Phenomena in relation to stomatal opening. V. Time and pH relations in amylase activity. — *Proc. roy. soc. Edinburg* 61: 254. 1942.
- Stälfelt, M. G.: Über die Wirkungsweise der Infiltrationsmethode von Molisch und einige Versuche mit derselben. — *Sv. Bot. Tidskr.* 10: 37. 1916.
- Die photischen Reaktionen im Spaltöffnungsmechanismus *Flora N. F.* 21 235 1927.
- Die Abhängigkeit der Spaltöffnungsreaktionen von der Wasserbilanz. — *Planta* 8: 287. 1929.
- Der stomatäre Regulator in der pflanzlichen Transpiration. — *Planta* 17: 22. 1932.
- Steinberger, Anne-Luise: Über Regulation des osmotischen Wertes in den Schliesszellen von Luft- und Wasserspalten. — *Biolog. Zentralbl.* 42: 405. 1922.
- Thomas, J. B.: Experiments on the water household of tropical plants. V. Water balance and stomatal movements in *Cissus sicyoides* L. — *Ann. Bot. Gardens, Buitenzorg* 51: 167. 1949.
- Went, F. W.: Plant growth under controlled conditions. — *Amer. J. bot.* 31: 597. 1944.
- Williams, W. T. & Barrett, F. A.: The Effect of external factors on Stomatal starch. — *Physiol. Plant.* 7: 298. 1954.
- Yin, H. C. and Tung, Y. T.: Phosphorylase in guard cells. — *Science* 108: 87. 1948.
- Yemm, E. W. & Willis, A. J.: Stomatal movements and changes of carbohydrate of *Chrysanthemum maximum*. — *New Phytologist* 53: 373. 1954.

Studies on the Browning and Blackening of Plant Tissues. IV. Chlorogenic Acid in the Leaves of *Nicotiana Tabacum*

By

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Introduction

Leaves of many plants undergo a colour change into brown or black when heated to about 60° C or mechanically injured. Such blackening usually takes place when they encounter heavy frost in early winter. Of such plants, we already reported *Aucuba japonica* (1), *Stizolobium Hassjoo* (2), and *Dahlia variabilis* (3). Leaves of *Aucuba japonica* contain a glycoside aucubin as a sole blackening agent. This glycoside consists of aucubigenin and glucose, and this aglycone is very liable to spontaneous oxidation in the air giving rise to a black substance of unknown structure. A glucosidase present in the leaves reacts with aucubin when the leaves are killed by higher or lower temperatures. In the case of *Stizolobium Hassjoo*, the matter is quite different. All parts of this plant contain dihydroxyphenylalanine (dopa), and this substance undergoes oxidation with a polyphenoloxidase which is also present in the tissues, when injury takes place. In the leaves of *Dahlia*, the substance responsible for the blackening is chlorogenic acid, which is oxidized by a polyphenoloxidase accompanying it.

It would be too hasty to conclude from the above findings that these three substances are equally distributed in higher plants, because Politis (4) showed a considerably wider distribution of chlorogenic acid in higher plants. On the contrary, the presence of aucubin (5) seems to be restricted, besides to *Aucuba* (Cornaceae), to some plants of *Eucommiaceae*, *Garry-*



Figure 1. Dark brown spots and rings produced on leaves of *Nicotiana glauca* («Bright yellow»), either by heating with a lighted cigarette tip or by injury. Spots No. I, II, and III are those produced by injury while handling.

aceae, Globulariaceae, Loganiaceae, Orobanchaceae, Plantaginaceae, and Scrophulariaceae, and dopa has been found only in three genera, *Stizolobium*, *Mucuna*, and *Vicia* of Leguminosae (cf. 2).

Leaves of *Nicotiana glauca* have been known to become dark brown when injured during tobacco processing. We have found also that the browning occurs on heating the leaves to 60° C. Death-ring formation by contact with a lighted cigarette tip on the leaf surface after Molisch (6) was also observed (Figure 1). We have now investigated its cause and found also in this case chlorogenic and caffeic acids as the principal agents responsible for the colour change induced by a polyphenoloxidase. Besides these acids, a flavonol glycoside, rutin, was present as is well known, but it is not to be regarded as the principal cause of the browning, although it possesses 3,4-dihydroxy group on the side phenyl ring, because the polyphenoloxidase does not attack it. This report deals with the results of our studies hitherto made of how to explain these facts.

Experimental part

1) Material

Leaves of *Nicotiana glauca* were supplied to us in 1953 and 1954 by the courtesy of the Hatano Tobacco Experiment Station of the Japan Monopoly Corporation. The varieties were «Bright Yellow», «Matsukawa» (an old variety raised in Japan), «White barley», and «Turkish Xanthi». Also an unknown variety of *Nicotiana rustica* was used.

One hundred g. of fresh leaves, the middle veins and petioles cut off, were crushed in a blender with 80 per cent ethanol, and the whole was heated on a water-bath. The extraction was repeated several times, renewing ethanol each time, until ferric chloride gave no more green coloration with the extract. The ethanolic extract was concentrated *in vacuo*, the residue was treated with 100 ml. of water and allowed to stand a while. After filtration the solution (A) was put in a refrigerator, and after several days standing, rutin almost completely precipitated. The filtrate of rutin (B) was several times extracted with ether, and chlorogenic acid remained in the aqueous layer (C). The ethereal layer (D) was distilled off, the residue was dissolved in petroleum ether, and this solution was extracted with 50 per cent ethanol. The ethanolic solution was condensed to 5 ml. and paper-chromatographed for the detection of caffeic acid.

2) *Paper-chromatography of phenolic substances, amino acids and sugars*

For paper-chromatography, 80 per cent phenol and butanol-acetic acid-water (4 : 1 : 1 or 4 : 1 : 2) were used as solvent throughout, and ascending method was applied with Whatman No. 1 filter paper at room temperature. Every time controls of chlorogenic acid, caffeic acid, and rutin were run with the test substances. For the detection of spots, ferric chloride, fluorescence with or without ammonia gas under ultraviolet light, BCG, and Hoepfner's reagent (7) were used.

Chlorogenic acid as control substance was isolated from de-fatted coffee beans after the method of Moores, McDermott, and Wood (8) except using isopropyl alcohol as extracting solvent. We used ethanol with good results. Chlorogenic acid was obtained as white crystals of m.p. 204° C.

Caffeic acid was obtained by hydrolyzing 4 g. of chlorogenic acid with 8 ml. of 10 per cent caustic potash solution at 30° C for 30 min. and neutralizing after cooling with 20 per cent sulfuric acid. Decolorized with charcoal and allowed to stand for 24 hrs., the solution precipitated crystals of caffeic acid. Lemon yellow crystals of m.p. 210—213° C.

Paper-chromatograms of amino acids and sugars were made by means of usual methods (2,3).

3) *Quantitative estimation of chlorogenic acid, caffeic acid, and rutin*

A) *Chlorogenic acid and caffeic acid.* — Aqueous solutions of pure chlorogenic acid and caffeic acid show absorption bands at 325 and 315 mμ, respectively, in ultraviolet (Figure 2). As there exists linear relationship between the concentration and $-\log T$ (T =per cent light transmission) of chlorogenic

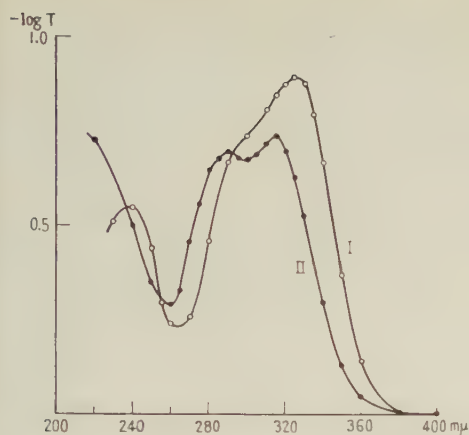


Figure 2.

Figure 2. Absorption curves of chlorogenic acid (I) and caffeic acid (II). Concentration, 5×10^{-5} M.

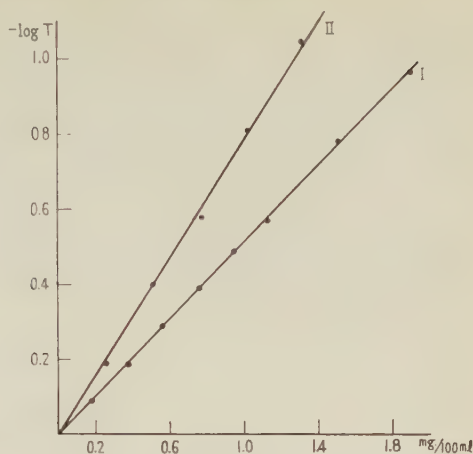


Figure 3.

Figure 3. Relationship between the concentration and $-\log T$ of chlorogenic acid (I) at 325 mμ and that of caffeic acid (II) at 315 mμ, respectively.

and caffeic acid, respectively (Figure 3), these acids are separated and spectrophotometrically estimated.

One ml. of the original extract, from which rutin had been removed, was filled up with water to 10 ml. and shaken twice with ether, the combined ether was distilled off, and the residue was dissolved in water and diluted to 10 ml. This procedure was repeated, while the aqueous layer was estimated at 315 mμ each time. When the value of caffeic acid, which at first became smaller and smaller, attained a constant value, a solution containing only caffeic acid was obtained. This ultimate solution is ten times dilute as compared with the initial extract (50 g. fresh leaves 50 ml.).

The aqueous layers, which were extracted with ether in above mentioned procedures, were combined and diluted to 100 ml. This solution was one hundred times dilute as compared with the initial leaf extract, and did not contain caffeic acid. Chlorogenic acid was estimated quantitatively in this solution.

All these solutions in the above procedures must have pH between 5 and 3. Otherwise the pH has to be adjusted to this value with acetic acid or caustic potash. Between these values, the absorptions at 325 and 315 mμ of chlorogenic acid and caffeic acid, respectively, did not vary.

As a control, the initial leaf extract was treated with lead acetate to

remove chlorogenic and caffeic acid, and estimated at the wave-length 325 and 315 m μ .

For this procedure, 4 ml. of the initial leaf extract were mixed with 0.8 ml. of a saturated lead acetate solution, 0.4 ml. of 10 per cent caustic potash and 0.8 ml. of water, (the whole volume was 6 ml.) and allowed to stand 1 hour under occasional shaking. This mixture showed pH 8—9 and all of chlorogenic and caffeic acid was completely precipitated.

From the filtrate of the lead precipitate 3 ml. were taken and mixed with 0.2 ml. of 10 per cent acetic acid and 0.8 ml. of water (the whole volume became 4 ml.). This solution is two times more dilute than that before the treatment with lead acetate, and showed pH about 4. All reagents used in this procedure proved to have no effect upon the absorption.

Chlorogenic acid was also estimated after the method of Moores, McDermott, and Wood (8) with iodometric oxidation method.

B) *Rutin*. — Fresh leaves were freed from the middle veins and petioles and 50 g. of them were crushed in 150 ml. of 80 per cent ethanol and extracted on a boiling water-bath. The extraction was repeated further two times with renewed ethanol. The combined ethanolic extract was condensed to 50 ml. in vacuo and allowed to stand a week in a refrigerator, when all of rutin separated in crystals. The precipitate of rutin was filtered, followed by drying at 110° C and weighing. Of the filtrate, chlorogenic acid and caffeic acid were quantitatively estimated.

4) *Quantitative estimation of ascorbic acid*

Reduced form of ascorbic acid in the leaves was estimated by the method of Fujita and Ebihara (9). Five g. of fresh leaves were crushed in a mortar with 20 ml. 5 per cent metaphosphoric acid and mixed with 25 ml. of water. After centrifuging, ascorbic acid was estimated in 2 ml. of the supernatant liquid.

5) *Preparation of crude polyphenoloxidase*

One kg. lamina of leaves (»Bright yellow«), taken just before yield from topped tobacco plants, was crushed in a blender and extracted with 1 l. water. The extract was squeezed through several-fold gauze and the liquid was then centrifuged (3000 r.p.m.: 5 min.). The reddish brown supernatant liquid (1.5 l.) was mixed slowly with 1.7 l. acetone, and the precipitate was centrifuged. The mother liquor of this precipitate did not contain polyphenoloxidase, because the precipitate which is formed upon addition of acetone did not show any polyphenoloxidase activity. The precipitate was

suspended in 50 per cent acetone and, after centrifuging, dried in a vacuum desiccator. Seventeen g. of dark brown powder were obtained. Above procedures were done at 0—5° C. For the estimation of the enzymatic reaction by a colorimetric method, the enzyme was further purified by repeated treatment with acetone and the less coloured preparation was used for the experiments.

Polyphenoloxidase preparation, which was obtained from white tissue powder, prepared from leaves by extraction with ethanol, proved to have a considerably lower activity.

6) *Estimation of polyphenoloxidase*

One ml. of 10^{-2} M substrate solution was mixed with 0.5 ml. of McIlvaine's buffer solution (pH 6.8) and 1 ml. of 0.5 per cent enzyme solution at 30° C for 30 min. to 5 hours, and it was tested whether the coloration due to oxidation of various substrates would occur.

7) *Estimation of polyphenoloxidase activity*

The activity of polyphenoloxidase was estimated by the following method. Three ml. of a solution of polyphenoloxidase (0.1 per cent) or homogenate of the leaves in McIlvaine's buffer solution were prepared and the increase in absorption at 480 mμ was estimated by a spectrophotometer every one min. within 5—10 min., immediately after 0.5 ml. of 0.1 per cent catechol solution was added. As control, a mixture of enzyme solution and 0.5 ml. of water was used. The temperature was held at 30° during the experiment. The activity of the enzyme was compared after the method of Ponting and Joslyn (10) by $-\log T/\text{min.}$

8) *Estimation of the polyphenoloxidase activity in the fresh leaves*

Thirty five g. of fresh leaves (water content 30 g.) were crushed in a blender with 120 ml. of McIlvaine's buffer solution (pH 6.8), held at slightly above 0° C. The mixture was squeezed through four-fold gauze and centrifuged at 0° C for 5 min. (1.5×10^4 r.p.m.). Ten ml. of the supernatant liquid was warmed to 30° C and the browning was electrophotometrically estimated.

When other substrates than catechol, for example chlorogenic acid, were used, the decrease in the quantity of the substrate was estimated for the activity.

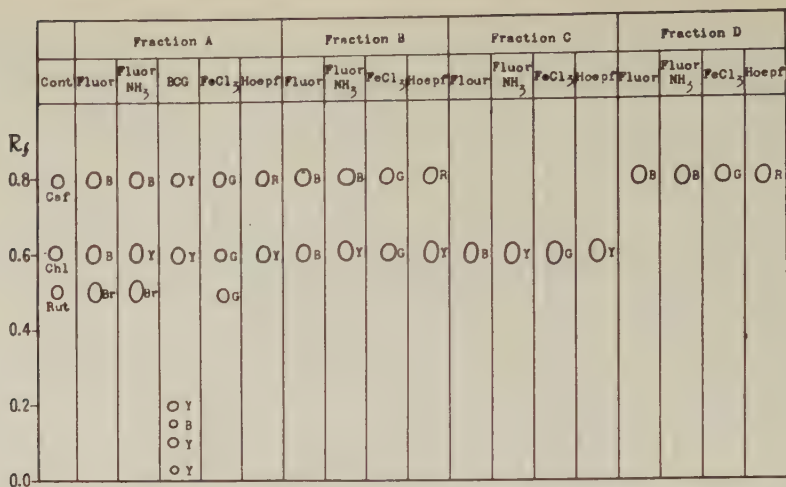


Figure 4. *Chromatograms of tobacco leaf extracts.* Cont, control, Fluor, fluorescence under ultraviolet light, fluor. NH_3 , fluorescence under ultraviolet light after treatment with ammonia gas, BCG, colour with BCG, FeCl_3 , colour with ferric chloride, Hoepf, colour with Hoepfner's reagent.

B, blue, Y, yellow, G, green, R, red, Br, brown.

Results and discussion

As phenolic substances, chlorogenic acid, caffeic acid, and rutin were found in the extract of the fresh leaves by paper-chromatography. The R_f values of the spots of these substances and the colours given by various reagents are shown in Table 1, and they well coincided with those of control substances. The R_f values, however, often deviated from those of the controls. In those cases, large scale chromatograms were made, from which each spot was isolated and again chromatographed, when the R_f value became quite identical with the controls. Therefore it is clear that the spots I, II and III are rutin, chlorogenic acid, and caffeic acid, respectively (Figure 4, Table 1).

Dopa and tyrosine could not be detected by any means.

Table 1. R_f values and reactions of the spots I, II and III.

Control	Spot	R_f (butanol: acetic acid-water 4:1:1)	Fluorescence	Fluorescence with NH_3	Colour with ferric chloride	Colour with Hoepfner's reagent
Caffeic acid	III	0.80	Blue	Blue	Green	Red
Chlorogenic acid	II	0.61	Blue	Yellow	Green	Yellow
Rutin.....	I	0.51	Brown	Blue	Green	—

Table 2. The contents of rutin, chlorogenic acid, and caffeic acid.

Material	Variety	Condition of leaves	Rutin ¹	Chlorogenic acid ¹
Collected June 21, 1954				
<i>Tabacum</i>	Bright yellow	Younger leaf (upper part of stem)	0.28	0.60
»	» »	Adult leaf (middle part of stem)	0.20	0.40
»	» »	Adult leaf (lowest part of stem)	0.11	0.26
»	White barley	Adult leaf (middle part of stem)	0.05	0.26
»	Matsukawa	» » » » » »	0.19	0.26
»	Xanthi	» » » » » »	0.09	0.26
<i>rustica</i>	An old form	» » » » » »	0.19	0.17
Collected Aug. 2, 1954				
<i>Tabacum</i>	Bright yellow	Younger leaf of a plant, not topped	0.36	0.76
»	» »	Adult leaf on the middle part of stem of a plant, not topped	0.24	0.72
»	» »	Adult leaf of the middle part of stem of a plant topped	0.20	0.56

¹ g. per 100 g. fresh leaf.

The contents of these three phenolic substances in the leaves differed according to varieties and difference in the position of insertion, and seemed to depend upon the vegetation period and cultivation condition even in the same variety (Table 2).

The quantity of chlorogenic acid varied in a range of 60.0 mg. per 100 g. fresh younger leaves and 0.26 mg. in adult leaves taken June 21. It grew, however, to some extent in the leaves taken Aug. 2, and at the same time the difference in quantity became smaller, that is, 0.76 mg. per 100 g. fresh younger leaves and 0.56 mg. per 100 g. fresh adult leaves. The quantity of rutin was in general pretty high in tobacco leaves, and the younger the leaves were, the higher the content of rutin was. However, no or a very small quantity of rutin was found in »White Barley» (Table 2).

As to the amount of caffeic acid in tobacco, there was found far smaller amount of it than chlorogenic acid.

Nine amino acids were found in the intact green leaves as well as in those parts of leaves which turned into brown by heating to 60° C. Those are proline, leucine, valine (or lysine), alanine, glycine, asparagin, serine, aspartic acid, and an unknown amino acid (Figure 5). It is very interesting that no change was found in amino acids before and after browning of the leaves, although such was the case with Dahlia leaves (3). As regards this difference, we will treat it at another opportunity.

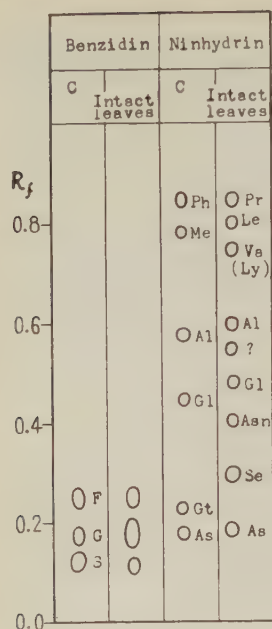


Figure 5. Paper chromatograms of sugars and amino acids in green intact tobacco leaves. Sugars are developed with benzidine reagent, and amino acids with ninhydrin. F, fructose, G, glucose, S, sucrose, Ph, phenylalanine, Me, methionine, Al, alanine, Gl, glycine, Gt, glutamic acid, As, aspartic acid, Pr, proline, Le, leucine, Va, valine, Ly, lysine, Asn, asparagine, Se, serine.

The sugars that were found are fructose, glucose, and sucrose, as is generally the case with leaves of most plants.

All of these three phenolic substances, rutin, chlorogenic acid, and caffeic acid, have an orthodihydroxyphenyl group, which might be apt to undergo oxidation with polyphenol oxidases in the presence of the air. It was then examined, as in previous studies, how these had changed in the leaves which had turned black. Ten g. of fresh leaves were heated to 100° C for 5—10 min. and another 10 g. of fresh leaves were heated to 60° C for 5—10 min. The former leaves remained green, seemingly showing no colour change, but the latter changed black owing to the oxidation of phenolic substances. Both of them were extracted as usual and the extracts were paper-chromatographed, with the results, that in the leaves which had changed into black, chlorogenic and caffeic acid had completely disappeared, although amino acids and sugars did not show any change. These findings are quite the same as in *Dahlia* leaves, and the substances responsible for blackening of the leaves must be chlorogenic and caffeic acid also in this case.

In order to determine exactly the oxidizability of various phenolic substances by the polyphenoloxidase of the tobacco leaves, twelve substances were examined (Table 3). Of these substances, 7 substances including chlorogenic and caffeic acid were oxidized by the enzyme, and the former acid gave a green colour, which became reddish brown and ultimately dark

Table 3. Oxidizability of some phenolic substances by means of polyphenoloxidase of tobacco leaves.

Phenols	Colour change into brown
Rutin	—
Chlorogenic acid	+
Caffeic acid	+
Tobacco leaf extract	+
Dopa	+
Tyrosine	—
Catechol	+
Pyrogallol	+
Hydroquinone	+
Resorcinol	—
<i>p</i> -Cresol	white precipitate
<i>p</i> -Phenylenediamine	+
Aucubin	—

brown, while the latter became directly reddish brown turning dark brown. On the contrary, rutin did not show any tendency to undergo appreciable colour change. Hence it is to be regarded as not oxidizable by this polyphenoloxidase.

As was reported in the case of *Dahlia* leaves, the younger the leaves, the stronger the blackening reaction when heated to 60° C, although the content of chlorogenic acid did not show any appreciable difference (Table 2, 4). The activity of the polyphenoloxidase also proved to show no considerable difference between younger or older leaves (Table 4). In a case, which was tested on Aug. 24, 1953, the polyphenoloxidase activity was rather greater in older leaves than in younger ones. This fact may suggest the presence of some substance which can affect the reaction taking place between phenolic substances and polyphenoloxidase.

We confirmed previously (2,3) in the leaves of *Stizolobium Hassjoo* and *Dahlia variabilis*, that the formation of dark brown melanoid substance was inhibited by the reduced form of ascorbic acid. Ponting and Joslyn (10) are of the same opinion, while Baruah and Swain (11) concluded that polyphenoloxidase undergoes a marked denaturation in the presence of ascorbic acid. As we do not at present aim at analyzing this point, we will return to

Table 4. Negative correlation between the activity of the polyphenoloxidase and the grade of death-ring formation.

Material (Aug. 2, 1954)	Activity of the enzyme (—logT/min.)	Time required for the full formation of death-ring (Seconds)
Younger leaf of an untopped plant ...	0.40	10
Adult leaf of an untopped plant	0.40	35
Adult leaf of a topped plant	0.44	60

Table 5. Relation between the quantity of chlorogenic acid oxidized by the polyphenol-oxidase and that of ascorbic acid.

Material (Aug. 24, 1953)	Quantity (mg.) of chlorogenic acid which was oxidized out of 70 mg by the polyphenoloxidase	Browning	Ascorbic acid (mg. per 100 g. fresh leaf)
Younger leaf	38	+++	19
Adult leaf on the middle part of stem	43	++	31
Adult leaf on the lower part of stem	49	+	—

As the enzyme preparation, an extract of 1.7 g leaf powder with 30 ml. water (50 hrs.) was used.

10 ml. enzyme solution, 70 mg. chlorogenic acid in 30 ml. water, 5 ml. McIlvaine's buffer solution (pH 6.8). Total 45 ml.

After standing 50 hrs. at 30 °, the remaining chlorogenic acid was estimated by the method of Moores, McDermott and Wood (8).

the subject at another opportunity. Moreover, the blackening reaction took place very easily in those plants containing a lesser amount of reduced form of ascorbic acid, and very difficultly so in those containing greater amounts of ascorbic acid (for instance, *Diospyros Kaki* Thunberg (11), *Thea sinensis* L. (11), *Camellia japonica* L. (11), *Brassica oleracea* L. (11), *Lycopersicon esculentum* Miller (11), *Fragaria chiloensis* Duchartre var. *ananassa* Bailey (11), *Iris gracilipes* A. Gray (12), *I. tectorum* Maxim. (12), *Capsicum annuum* L. var. *acuminatum* Fingerhuth (12), etc.). The same has proved to be true also of the tobacco leaves (Table 5). This inhibition continued to take place as long as reduced form of ascorbic acid was available in the cell, while oxygen uptake went on parallel to the available quantity of ascorbic acid and the more ascorbic acid was present, the weaker the colour produced was. In fact, the ascorbic acid content was 19 mg. per 100 g. fresh leaves in younger leaves and attained 31 mg. per 100 g. fresh leaves in adult, sometimes yellowish green leaves.

Summary

1) The presence of three phenolic substances, chlorogenic acid, caffeic acid, and rutin, was confirmed in the leaves of tobacco by paper chromatography. Rutin was also isolated in substance.

2) A polyphenoloxidase present in the leaves proved to provoke a dark brown colour when it attacked chlorogenic and caffeic acid, but rutin was not oxidized.

3) In tobacco leaves in which blackening was remarkable, the content of reduced form of ascorbic acid was low, and *vice versa*.

We are grateful to Dr K. Okuma, the Hatano Tobacco Experiment Station, for giving us a lot of tobacco leaves. The cost for this study was defrayed partly with a stipendium given us by the Japan Monopoly Corporation and partly with the Stipendium for the Advancement of Scientific Research, given to one of us (S. H) by the Ministry of Education.

References

1. Hattori, S. & Kurihara, M.: Studies on death-ring. I. On an enzyme hydrolyzing aucubin in the leaves of *Aucuba japonica*. — Misc. Rep. Res. Inst. f. Nat. Resources. Nos 17—18: 163. 1950.
2. — & Shiroya, M.: Studies on the browning and blackening of plant tissues. II. On the interaction of dopa and a specific oxidase in the leaves of *Stizolobium Hassjoo*. — *Physiol. Plant.* 8: 63. 1955.
3. Shiroya, M. & Hattori, S.: Studies on the browning and blackening of plant tissues. III. Occurrence in the leaves of *Dahlia* and several other plants of chlorogenic acid as the principal browning agent. — *Physiol. Plant.* 8: 358. 1955.
4. Politis, J., Sur la distribution de l'acide chlorogénique dans la famille de Solanacées et dans les organes de ces plantes. — *Compt. rend. Acad. Sci. Paris* 226: 692. 1948.
5. Trim, A. R. & Hill, R.: The preparation and properties of aucubin, asperuloside and some related glycosides. — *Biochem. J.* 50: 310. 1952.
6. Molisch, H.: *Botanische Versuche ohne Apparate*. Jena. 53. 1931.
7. Hoepfner, W.: Zwei neue Reaktionen für Kaffeesäure und Chlorogensäure. — *Chem. Ztg.* 56: 991. 1932.
8. Moores, R. G., McDermott, D. L. & Wood, T. R.: Determination of chlorogenic acid in coffee. — *Anal. Chem.* 20: 620. 1948.
9. Fujita, A. und Ebihara, T.: Kolorimetrische Bestimmung von Vitamin C mittels Phospho-18-wolframsäure. I. Bestimmung der reducierten Form von Vitamin C. — *Biochem. Z.* 290: 182. 1937.
10. Ponting, J. D. & Joslyn, M. A.: Ascorbic acid oxidation and browning in apple tissue extracts. — *Arch. Biochem.* 19: 47. 1948.
11. Baruah, P. & Swain, T.: The effect of *l*-ascorbic acid on the in vitro activity of polyphenoloxidase from potato. — *Biochem. J.* 55: 392. 1953.
12. Fujita, A. & Ebihara, T.: Über die Verbreitung des Vitamins C in tierischen und pflanzlichen Geweben. I. — *Biochem. Z.* 290: 201. 1937.
13. Sugawara, T.: Studies on the formation of ascorbic acid in plants. 3. Relation between the accumulation of ascorbic acid and the carbohydrate contents in plants. — *Jap. J. Bot.* 11: 147. 1941.

Comparative Effects of Ammonium and Nitrate Ions on the Growth and Composition of *Hevea brasiliensis*

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It is known that field application of nitrogen containing fertilisers may greatly improve the yield of rubber obtained from young stands of *Hevea brasiliensis* and maintain the yield of more mature trees (Haines and Guest, 1936; Haines, 1946). Grantham (1924, 1927, 1930) obtained growth and yield responses by applying nitrogen either as ammonium sulphate or sodium nitrate to young mature rubber trees but his data do not provide a direct quantitative comparison between the relative efficiency of these two nitrogen sources; according to Vollema (1931), however, both sources of nitrogen may give equivalent yield responses from mature rubber trees. The concentration of rubber found in very young nitrogen deficient *Hevea* seedlings, grown in sand culture, may be smaller than that of healthy plants but as development proceeds a reversal occurs and the healthy plants contain a smaller concentration of rubber but grow much larger than the deficient plants (Bolle-Jones 1954, b); a similar reduction in the concentration of rubber with increased amounts of nitrogen may occur in guayule grown in soil (Benedict 1950).

A differential effect on rubber concentration of the guayule plant, according to the form in which nitrogen was supplied was noted by Bonner (1944) who found that a high nitrate type of nutrient gave the highest concentration of rubber in the leaves; this concentration decreased as the proportion of ammonium ions in the nutrient increased. Okankenko and Bershtein (1950) and Pinevic (1952) concluded that the amount of rubber produced by koksaghyz plants depended upon the form in which nitrogen was supplied.

Consequently it may be inferred from the literature that the application

of nitrogen will increase the growth and the amount of rubber yielded by rubber producing plants but the form in which the nitrogen is applied may affect the total yield and, perhaps, the concentration of rubber found within the plant.

It was desirable to establish with certainty (a) whether *Hevea brasiliensis* supplied with a high proportion of ammonium nitrogen would grow as well, or better, than if supplied with a high proportion of nitrate nitrogen, (b) the differential effect, if any, of these two nitrogen sources on the chlorophyll, rubber and mineral concentrations of the plant. A knowledge of the tolerance to high ammonium nitrogen supplies under varied conditions of calcium supply was also sought, bearing in mind the usual very acid condition of Malayan soils and their relatively low exchangeable calcium status.

In a factorial experiment designed to investigate these problems Tjirandji 1 seedlings were grown in pot-sand-culture at varying levels of ammonium nitrogen and nitrate nitrogen supplies either in the presence of a very low or a normal supply of calcium. Observations on the growth and composition of the plants were made at intervals during the course of the experiment.

Experimental

Design of Experiment

There were 18 treatments derived from all combinations of the following levels of NH_4^+ (Am), NO_3^- (N) and Ca^{++} ions.

Am ₁	1.0 m.eq./l.	N ₁	1.0 m.eq./l.	Ca ₁	0.5 m.eq./l.
Am ₂	3.0 m.eq./l.	N ₂	3.0 m.eq./l.	Ca ₂	4.0 m.eq./l.
Am ₃	5.0 m.eq./l.	N ₃	5.0 m.eq./l.		

Other nutrients were kept constant, with the exception of sodium and sulphate, at the following levels: (in m.eq./l.) Mg^{++} 2.0; K^+ 3.0; PO_4 3.0; Fe^{+++} 1.0; Mn^{++} 0.04; Cu^{++} 0.002; Zn^{++} 0.002; BO_3^- 0.018; Mo^{VI} 0.0012. The Na^+ level varied from 1.0 to 2.5 m.eq./l., and the SO_4^{--} level from 3.0 to 13 m.eq./l. (increasing with Am level but decreasing with N level). Tap-water was used in the preparation of the nutrients.

Cultural And Sampling Methods

Each treatment was carried out in duplicate; each plot contained three pots in each of which twelve Tjirandji 1 (selfed) seeds were sown on 15th December 1953. The bitumen painted pots and the sand used have been described (Bolle-Jones 1954, a), the sand was washed with cold 2 % hydrochloric acid (w/v) for four days

perior to being leached with calcium nitrate solution until free of acid. Complete plants were removed from each pot and leaves taken for analysis on the following dates: 17 May (1954), 27 July, 13 September, 22 November (final sampling).

At each sampling fully expanded leaves were removed from a fixed whorl position (mainly second whorl). The midribs were excised and discarded; the laminae were analysed for chlorophyll, nitrogen and mineral concentrations and, when possible, for their rubber concentration. For the earlier samplings the green part of the stems was bulked with the petioles from each plot prior to drying, milling and rubber extraction; for the September and November samplings the rubber concentration of the petioles was estimated separately.

During the course of the experiment the pH and the ammonium and nitrate nitrogen concentrations of the added nutrients and of the leachates, issuing from the drainage hole of each pot, were measured. To collect the leachates 1 litre of the respective nutrient was added to each pot (each of which had received a similar amount 24 hours previously) in a treatment plot and the first 25 ml. of effluent which issued from each pot was collected and bulked with the other leachates to give a 75 ml. plot sample. Preliminary trials had shown that the composition of any 25 ml. aliquot collected within the first 100 ml. of effluent did not vary significantly according to whether it was the first or last fraction taken within this 100 ml. volume.

Analytical Procedures

All the analytical procedures employed have been described (Bolle-Jones 1954, a) with the exception of the estimation of rubber in the laminae and petioles and of ammonia-, nitrate-, amide- and amino-, nitrogen fractions in the laminae; these are briefly described below.

Rubber: 5 g. of milled (40 mesh screen) dry laminar (or petiolar) tissue were extracted with 50 ml. of a 1 % trichloroacetic acid solution in benzene, by mechanical shaking, for 15 minutes. A 20 ml. aliquot of the supernatant liquid was brominated for 100 minutes as described by Meeks, Crook, Pardo and Clark (1953); 125 ml. of 95 % ethyl alcohol were added and the solution allowed to stand for 2 hours. The deposited rubber bromide was filtered off and treated subsequently as described by Meeks *et alii*. Based on the percentage of bromine found by analysis in the rubber bromide a conversion factor of 0.274 was used to convert weights of bromide to the hydrocarbon. The method permitted a recovery of 75 % of the pure rubber added to the dry laminar powder prior to extraction.

Soluble Nitrogen Constituents — Extraction: 22 ml. of hot water (60—70° C) were added to 1 g. of powdered laminae (rapidly oven dried from the fresh state at 70—80° C) and the mixture heated, with constant stirring, on a boiling water bath for 10 minutes. The suspension was allowed to cool before the addition of 2.5 ml. of 0.25 M. lead acetate solution and the total volume was made up to 30 ml. with water. After standing for 30 minutes the suspension was centrifuged to deposit the voluminous laminar residue. To 20 ml. of the supernatant liquid 12.5 ml. of 0.1 M, sodium carbonate solution were added (to precipitate the lead) and the solution again centrifuged. The clear supernatant liquid was filtered through No. 42 Whatman filter paper and stored in glass stoppered containers at 10° C until analysed for nitrate-nitrogen, ammonia-nitrogen, amide-nitrogen and for the free amino-acid nitrogen.

Nitrate-Nitrogen: To an aliquot, equivalent to 0.06 g. of dried laminae, 0.1 ml. of

1 % potassium hydroxide and 1 ml. of Analar hydrogen peroxide (100 vols.) were added and the solution simmered on a steam bath for 2 hours to oxidise traces of coloured organic matter; if the extract remained coloured a further addition (0.2—0.5 ml.) of peroxide was necessary. The solution was evaporated almost to dryness, 1—2 ml. of water added and the solution again evaporated almost to dryness; this process was thrice repeated to eliminate all traces of peroxide. Finally the residue was gently dried on a steam bath for an additional 30 minutes, leaving a perfectly white residue. After cooling, 2 ml. of phenol disulphonic acid were added followed, 5 minutes later, by 25 ml. of water; the solution was transferred to a graduated flask, an excess of concentrated ammonia solution (5 ml.) added and the solution rapidly cooled. The solution was made up to 50 ml. with water, centrifuged and the colour intensity measured. This procedure based on a method described by Burström (1942) enabled 90 to a 110 % of the nitrate added to dry laminae (prior to water extraction *et. seq.*) to be recovered.

Ammonia-Nitrogen and Amide-Nitrogen: An aliquot of the clarified aqueous extract equivalent to 0.4 g. of dried laminae was distilled under reduced pressure at 40° C in the presence of a pH 10 borate buffer; this removed the ammonia-nitrogen which was collected and estimated. Concentrated sodium hydroxide (40 %) was then added to the distillation flask and the amide nitrogen removed by steam distillation at 100° C. These procedures (described fully by Varner, Bulen, Vanecko and Burrell, 1953) permitted recovery of 77 % of the ammonium nitrogen added to dried lamina (prior to water extraction) and of approximately 80 % of the amide nitrogen (contained in either glutamine or asparagine) similarly added.

Free Amino-Acid Nitrogen: To an aliquot of the aqueous extract equivalent to 0.06 g. of dry laminae, ninhydrin was added and the liberated α amino-nitrogen was determined, after the addition of potassium hydroxide, as ammonia. For a similar volume of extract a parallel estimation was made, but without the addition of ninhydrin. This provided values for any interfering ammonia- or amide-, nitrogen, present and which could be applied as a correction factor to the value obtained in the presence of ninhydrin. The method, details of which are described by Sobel, Hirschman and Besman (1945), permitted a recovery of 70—80 % of the amino-nitrogen added as glycine or alanine to the dry laminae before aqueous extraction.

Results

For many of the numerical data it is not possible to present results obtained for each individual sampling date. Consequently, where the trends appeared similar, values for the same treatment replicate for each month were combined and are presented in the form of mean tables together with the relevant standard errors. For the statistical analysis 35 degrees of freedom only were considered rather than that number multiplied by the number of sampling dates.

Visual Observations

Stunted, pale, small leaved plants with yellow leaflets in the upper expanded whorls, characteristic of nitrogen deficiency, were seen in the



Figure 1. *Tjirandji 1* seedlings, 7 months old, low calcium supply. $Am_3 N_1 Ca_1$: $Am_1 N_1 Ca_1$: $Am_1 N_3 Ca_1$. (Middle): Weak stemmed, few leaved, nitrogen deficient plants. (left): High ammonium and (right) high nitrate plants — tall and vigorous.

$Am_1 N_1 Ca_1$ and $Am_1 N_1 Ca_2$ treatments early in the experiment. Similar symptoms were recorded later in the $Am_1 N_2 Ca_1$ and $Am_1 N_2 Ca_2$ plants (June) and in the $Am_2 N_1 Ca_1$ and $Am_2 N_1 Ca_2$ plants (November).

Plants which received the high levels of ammonium and calcium showed well marked symptoms of magnesium deficiency until May. Later (June) the symptoms appeared milder and, towards the end of the experiment, of little consequence.

The incidence of Bird's Eye Spot disease (*Helminthosporium heveae*), as measured by the number of lesions and of affected leaflets, appeared related to the nutrient level. Both ammonium and nitrate addition increased the incidence of the disease but calcium had the reverse effect. Whether the severity of the disease was related to the nutrient level is being further investigated in subsequent experiments.

The number of leaves borne per plant increased with ammonium or nitrate supply (Fig. 1): nitrate was more effective than ammonium. Increased calcium level usually increased the number of leaves produced at the higher levels of ammonium and nitrate supply but had little effect at the lower levels. The number of leaflets produced per whorl or flush also varied markedly with nitrate level and, to a lesser extent, with ammonium.

Increased ammonium or nitrate supplies increased the height of each plant; the overall effect of nitrate was greater than that of ammonium (Figure 1). The height difference between the Am_1 and Am_2 plants was much greater than that between the Am_2 and Am_3 . Increased calcium did not produce a consistent effect. The tallest plants in the experiment grew in the $Am_3 N_3 Ca_1$ treatment (270 cms.) and the shortest in the $Am_1 N_1 Ca_1$ treatment (141 cms.). Neither the number of leaves nor the height measurements are presented here.

Table 1. *Girth of stems and total dry weight of plant* (shoot+root). Girth measured at a height of 7 cms above sand level on 10.11.54 and dry weight taken on 22.11.54.

Level	Am ₁	Am ₂	Am ₃	Ca ₁	Ca ₂	Mean
<i>Girth (cms)</i>						
N ₁	5.4	7.5	7.2	6.6	6.7	6.7
N ₂	7.6	8.1	8.0	8.0	7.8	7.9
N ₃	8.7	(± 0.22) 8.8	8.6	(± 0.18) 8.5	8.9	(± 0.13) 8.7
Ca ₁	7.3	8.1	7.7			7.7
Ca ₂	7.2	(± 0.18) 8.1	8.2			(± 0.10) 7.8
Mean	7.2	8.1 (± 0.13)	7.9	7.7 (± 0.10)	7.8	7.8
<i>Dry weight (grams)</i>						
N ₁	141	351	334	278	272	275
N ₂	339	421	422	398	390	394
N ₃	439	(± 22) 486	498	(± 18) 456	492	(± 13) 474
Ca ₁	300	423	410			378
Ca ₂	313	(± 18) 416	426			(± 10) 385
Mean	306	419 (± 13)	418	378 (± 10)	385	381

Girth measurements of the stem were taken, towards the end of the experiment, at a height of 7 cms. above sand level. Both ammonium and nitrate addition increased the girth (Table 1) but whereas the N₃ plants possessed a larger girth than the N₂, the Am₃ Ca₁ plants possessed a slightly smaller girth than the Am₂ Ca₁. The overall effect of nitrogen given as nitrate was greater than if given as ammonium (Table 1). If the girth measurement can be regarded as an index of growth the observations suggested that the slight but not significant decrease in girth at the Am₃ Ca₁ level was due to a mild toxicity effect (which was not reflected in the height observations). No such suggestion of toxicity was obvious for nitrate at the levels used.

Dry Weight of Plant (Table 1)

Nitrate application produced a greater dry weight per plant response than did ammonium and was most beneficial when applied to the Am₁ plants. Nitrate addition produced a greater response when applied in the presence of the higher level of calcium but this interaction effect (N×Ca) was not significant. The dry weight values, recorded in November, showed

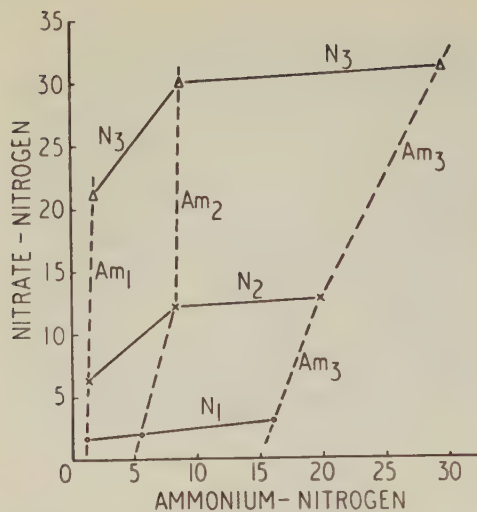


Figure 2. Ammonia -N and nitrate -N concentrations (expressed as p.p.m. nitrogen) of leachate. Overall means for July September and October values. Continuous lines denote effect of increasing ammonium supply; broken lines denote effect of increasing nitrate supply.

that an increase of ammonium supply from the Am₂ to the Am₃ level produced no significant overall response but that there were indications of a slight depressive effect in the Ca₁ treatments. The beneficial, but not significant, effect of calcium addition was only apparent at the Am₃ and N₃ levels; it produced no effect at the lower levels.

Plants which grew most occurred in the Am₂ N₃ Ca₂ (505 g.) and Am₃ N₃ Ca₂ (518 g.) treatments.

Leachate Composition

The mean pH and the nitrogen composition (in p.p.m.) of the nutrients were found to be as follows:

Level	pH	Ammonia-N	Nitrate-N	Level	pH	Ammonia-N	Nitrate-N
Am ₁ ..	5.3	11	39	N ₁ ...	5.2	39	13
Am ₂ ..	5.2	40	40	N ₂ ...	5.2	38	39
Am ₃ ..	4.9	65	39	N ₃ ...	5.0	39	66
S.E.	± 0.06	± 0.92	± 0.83	S.E.	± 0.06	± 0.92	± 0.83

Once the nutrient solutions had been in contact with the roots of the plants these values rapidly changed to the following:

Level	pH	Ammonia-N	Nitrate-N	Level	pH	Ammonia-N	Nitrate-N
Am ₁ ..	5.3	2	10	N ₁ ...	3.3	8	2
Am ₂ ..	3.4	8	15	N ₂ ...	3.8	10	10
Am ₃ ..	3.0	22	16	N ₃ ...	4.6	13	27
S.E.	± 0.09	± 0.97	± 1.9	S.E.	± 0.09	± 0.97	± 1.9

Table 2. *Per cent Rubber in Dried Petioles.* September and November samplings:
Mean values.

Level	Am ₁	Am ₂	Am ₃	Ca ₁	Ca ₂	Mean
N ₁	1.58	1.42	1.46	1.45	1.52	1.48
N ₂	1.37	1.44	1.32	1.43	1.32	1.38
		(± 0.11)		(± 0.09)		(± 0.06)
N ₃	1.55	1.42	1.28	1.51	1.33	1.42
Ca ₁	1.50	1.44	1.45			1.46
		(± 0.09)				(± 0.05)
Ca ₂	1.50	1.41	1.25			1.39
Mean	1.50	1.43	1.35	1.46	1.39	1.43
		(± 0.06)		(± 0.05)		

The pH of the leachate decreased as the ammonium level of the nutrient solution increased, while increased nitrate level had a smaller but opposite effect. As the ammonium level of the nutrient solution increased the nitrate concentration of the leachate increased, and as the nitrate level of the nutrient increased so did the ammonium concentration of the leachate. The former effect may have been partially caused by the nitrification of some of the nutrient ammonium-nitrogen to the nitrate form. This is illustrated in Figure 2 which shows an increase in nitrate concentration of the leachate (at any one nitrate level) as the ammonium level of the nutrient increased. The sharp increase of the nitrate concentration of the leachate at the N₂ and N₃ levels as the ammonium supply was increased from Am₁ to Am₂, and the absence of further increase from Am₂ to Am₃, suggested that at the higher N levels nitrification may proceed only to a limited extent and that this limit was related to the nitrate concentration.

Composition of Plant

Rubber. (Table 2). — Determinations of the rubber concentration of the stems, petioles and laminae were carried out at varying times during the experiment. These analyses revealed that an increase in either ammonium or nitrate levels generally decreased the concentration of rubber in the tissues examined. For the laminae and for the (bulked) stems and petioles the nitrate effect achieved bare significance; the ammonium effect was not usually significant. Calcium addition had little effect. Values for the petiolar rubber concentrations are presented in Table 2.

Figure 3 illustrates how the rubber concentration of the laminae declined as the total nitrogen concentration of the laminae increased.

Chlorophyll. (Table 3). — Addition of either ammonium or nitrate nitrogen

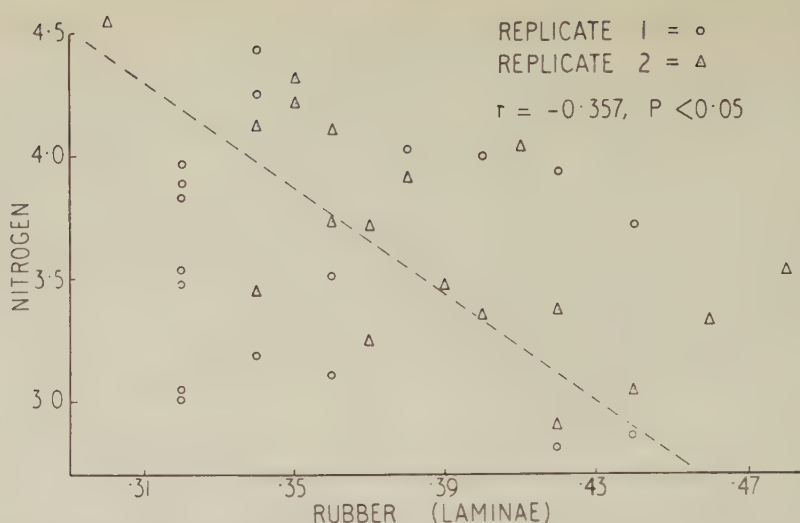


Figure 3. Variation of rubber concentration of laminae with total nitrogen concentration. (Results expressed as % of dry laminae). Means of September and November values.

Table 3. Chlorophyll and total nitrogen concentrations in laminae. May, July, September and November samplings. Mean values. Chlorophyll expressed as mg. per gram of dry lamina and nitrogen as % of dry laminae.

Level	Am ₁	Am ₂	Am ₃	Ca ₁	Ca ₂	Mean
<i>Chlorophyll</i>						
N ₁	5.51	6.54	7.87	6.91	6.37	6.64
N ₂	5.81	7.55	8.50	7.26	7.31	7.29
N ₃	6.86	(± 0.19) 7.41	8.61	7.77	(± 0.15) 7.48	(± 0.11) 7.62
Ca ₁	6.28	7.24	8.42			7.31
Ca ₂	5.84	(± 0.15) 7.09	8.23			(± 0.09) 7.05
Mean	6.06	7.17 (± 0.11)	8.32	7.31 (± 0.09)	7.05	7.18
<i>Nitrogen</i>						
N ₁	2.95	3.18	3.77	3.38	3.23	3.30
N ₂	3.04	3.47	4.04	3.54	3.50	3.52
N ₃	3.36	(± 0.049) 3.79	4.22	3.84	(± 0.040) 3.74	(± 0.028) 3.79
Ca ₁	3.16	3.49	4.10			3.58
Ca ₂	3.08	(± 0.040) 3.47	3.91			(± 0.023) 3.49
Mean	3.12	3.48 (± 0.028)	4.01	3.58 (± 0.023)	3.49	3.54

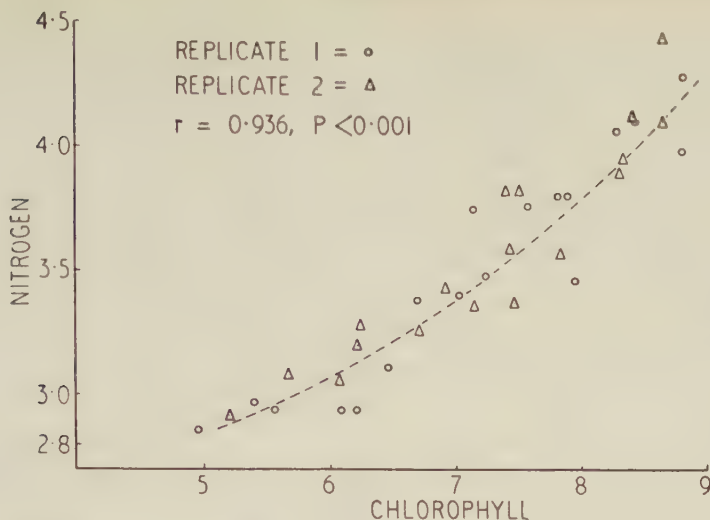


Figure 4. Variation of chlorophyll concentration of laminae with total nitrogen concentration. (Chlorophyll expressed as mgms. per gm. of dry matter and nitrogen as % of dry laminae). Means of May, July, September and November values.

increased the chlorophyll concentration of the laminae. This was reflected in the strong positive correlation ($r=0.94$) between the laminar total nitrogen and chlorophyll concentrations (Figure 4). Nitrogen added as ammonium was usually more efficient in increasing the chlorophyll concentration than if added as nitrate. The depressive effect of calcium level was barely significant but the correlation between the laminar chlorophyll and calcium concentrations was both highly significant and negative (Figure 5). The relationship was not so clearly defined as that which existed between nitrogen and chlorophyll but it was evidently of overall importance to the plant.

Nitrogen. (Tables 3, 4, 5). — Increased ammonium or nitrate supply increased the total nitrogen concentration of the laminae whereas calcium addition caused a small but significant decrease. It was interesting to note (see Figure 6) that, although both ammonium and nitrate nitrogen applications increased the total nitrogen of the laminae, it was only when that increased concentration was induced by nitrate addition that the girth of the plants consistently increased with nitrogen concentration of the laminae. A similar increase was observed at the N_1 level on the addition of ammonium but no appreciable increase at the higher N levels was noted.

The total nitrogen concentration was related to the ammonia-nitrogen, nitrate-nitrogen, amide-nitrogen and the free amino-acid nitrogen of the laminae in the manner shown in Figure 7. With the exception of nitrate

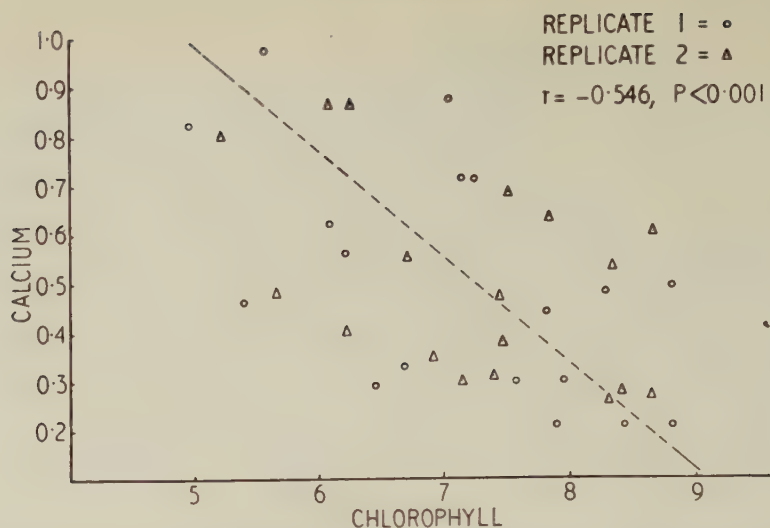


Figure 5. Variation of chlorophyll concentration of laminae with calcium concentration. (Chlorophyll expressed as mgms. per gm. of dry matter and calcium as % of dry laminae). Means of May, July, September and November values.

Table 4. Ammonia-N and nitrate-N concentrations in laminae. May, July, September and November samplings. Mean values — expressed as p.p.m. N of dry laminae.

Level	Am ₁	Am ₂	Am ₃	Ca ₁	Ca ₂	Mean
<i>Ammonia-N</i>						
N ₁	44	43	66	48	55	51
N ₂	45	57	77	54	65	60
		(± 4.6)			(± 3.7)	(± 2.6)
N ₃	45	84	100	75	77	76
Ca ₁	37	55	86			59
		(± 3.7)				(± 2.2)
Ca ₂	53	68	76			66
Mean	45	61	81	59	66	62
		(± 2.6)		(± 2.2)		
<i>Nitrate-N</i>						
N ₁	64	96	157	106	105	106
N ₂	77	125	179	120	133	127
		(± 10.0)			(± 8.1)	(± 5.7)
N ₃	114	176	214	183	153	168
Ca ₁	99	130	181			136
		(± 8.1)				(± 4.7)
Ca ₂	71	134	186			130
Mean	85	132	183	136	130	133
		(± 5.7)		(± 4.7)		

Table 5. *Amide-N and amino-N concentrations in laminae. May, July, September and November samplings. Mean values — expressed as p.p.m. N of dry laminae.*

Level	Am ₁	Am ₂	Am ₃	Ca ₁	Ca ₂	Mean
<i>Amide-N</i>						
N ₁	194	216	282	248	213	230
N ₂	208	237	300	263	234	248
N ₃	217	(± 15.8) 269	347	(± 12.9) 284	271	(± 9.1) 278
Ca ₁	218	253	324			265
Ca ₂	195	(± 12.9) 228	295			(± 7.5) 239
Mean	206	241 (± 9.1)	310	265 (± 7.5)	239	252
<i>Amino-N</i>						
N ₁	501	514	737	564	604	584
N ₂	510	588	957	677	693	685
N ₃	594	(± 40.2) 762	1079	(± 32.8) 854	770	(± 23.2) 812
Ca ₁	554	632	908			698
Ca ₂	515	(± 32.8) 611	940			(± 18.9) 689
Mean	535	622 (± 23.2)	924	698 (± 18.9)	689	694

these fractions appeared to be related to the total nitrogen by a curvilinear line. The total nitrogen/nitrate-nitrogen curve appeared, in the range examined, to be a straight line. The concentrations of amino-, amide-, and ammonia-, nitrogen, present decreased as the total nitrogen decreased but apparently were capable of either levelling off and reaching a constant concentration, or of decreasing much less rapidly in relation to the total nitrogen concentration.

Nitrogen added in the ammonium form was usually more efficient than nitrate in increasing the various soluble nitrogen fractions (Tables 4, 5). This suggested, in regard to the nitrate fraction, that much of the nitrogen entered the plants as the ammonium ion but that, in part, some of it was oxidised to nitrate within the plants — a possible detoxication mechanism.

Increased calcium supply significantly decreased the amide-nitrogen concentration of the laminae (Table 5) but increased that of the ammonia-nitrogen fraction (Table 4). A highly significant interaction between ammonium and calcium levels, on the laminar ammonia-nitrogen fraction was also evident (Table 4).

Phosphorus. (Table 6). — Increased ammonium or nitrate supply pro-

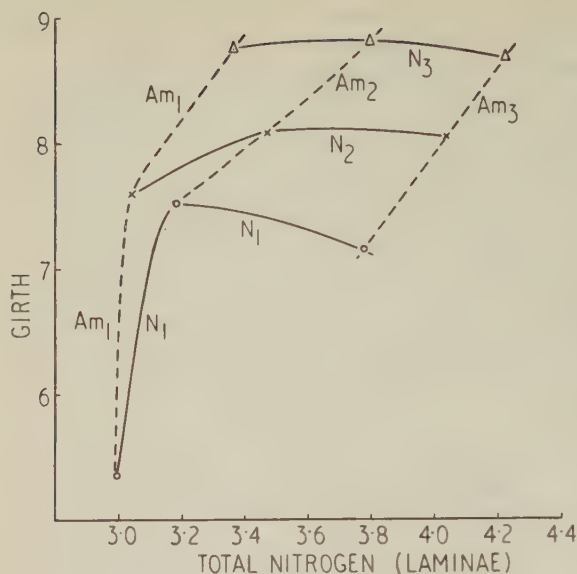


Figure 6. Relationship between girth measurement and nitrogen concentration of laminae. (Girth in cms. and nitrogen as % of dry matter). Overall mean values for November. Continuous lines denote effect of increasing ammonium supply; broken lines denote effect of increasing nitrate supply.

duced an overall decrease in the concentration of phosphorus in the laminae. While the depressive effect of ammonium supply was greatest at the N_1 level and of nitrate supply at the Am_1 level the opposite effects were recorded at the N_3 and Am_3 levels, respectively. This $Am \times N$ interaction effect was significant at the 0.1% level. Calcium addition did not produce any marked or consistent effect.

As the phosphorus concentration of the laminae decreased the free amino-acid nitrogen concentration increased, particularly at the Am_1 and N_1 levels (Figure 8). At the higher levels of nitrogen supply, increase in amino-nitrogen concentration became independent of phosphorus concentration.

Potassium. (Table 6). — The addition of either ammonium or nitrate nitrogen decreased the potassium concentration of the laminae. The overall effect of extra calcium supply was not significant but its effect varied with nitrate level to produce a significant $N \times Ca$ interaction effect (Table 6).

The potassium concentration of the laminae decreased almost linearly as the nitrogen concentration increased (Figure 9). This negative correlation was highly significant and was independent of the form in which the nitrogen was supplied to the plant.

The increase in ammonium or nitrate levels which resulted in a decreased potassium concentration also caused an increase in the concentration of free amino-acid nitrogen in the lamina (Fig. 8). As the potassium concentration of the laminae decreased the concentration of amino-nitrogen increased.

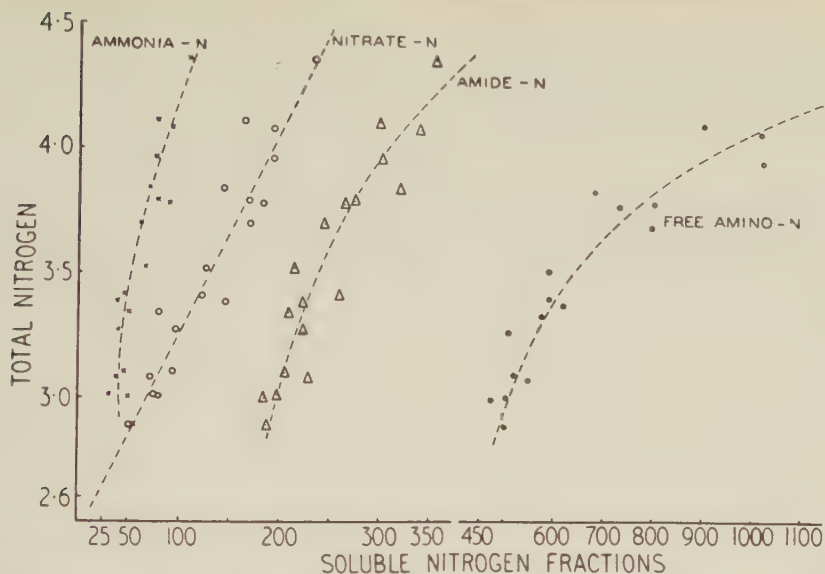


Figure 7. Relationship between total nitrogen concentration of laminae and: ammonia -N; nitrate -N; amide -N; amino-acid -N. (Total nitrogen expressed as % of dry matter and soluble fractions as p.p.m. N of dry laminae). Treatment means for May, July, September and November.

Table 6. Phosphorus and potassium concentrations in laminae. May, July, September and November samplings. Mean values — expressed as % of dry laminae.

Level	Am ₁	Am ₂	Am ₃	Ca ₁	Ca ₂	Mean
<i>Phosphorus</i>						
N ₁	0.50	0.34	0.29	0.38	0.37	0.38
N ₂	0.39	0.30	0.30	0.32	0.34	0.33
		(± .022)		(± .018)		(± .012)
N ₃	0.27	0.28	0.30	0.29	0.28	0.28
Ca ₁	0.38	0.31	0.30			0.33
		(± .018)				(± .010)
Ca ₂	0.40	0.31	0.29			0.33
Mean	0.39	0.31	0.30	0.33	0.33	0.33
		(± .012)		(± .010)		
<i>Potassium</i>						
N ₁	1.74	1.55	1.43	1.51	1.64	1.57
N ₂	1.60	1.33	1.28	1.37	1.44	1.41
		(± .044)		(± .036)		(± .025)
N ₃	1.54	1.21	1.14	1.35	1.24	1.30
Ca ₁	1.62	1.34	1.26			1.41
		(± .036)				(± .021)
Ca ₂	1.63	1.38	1.31			1.44
Mean	1.63	1.36	1.28	1.41	1.44	1.42
		(± .024)		(± .021)		

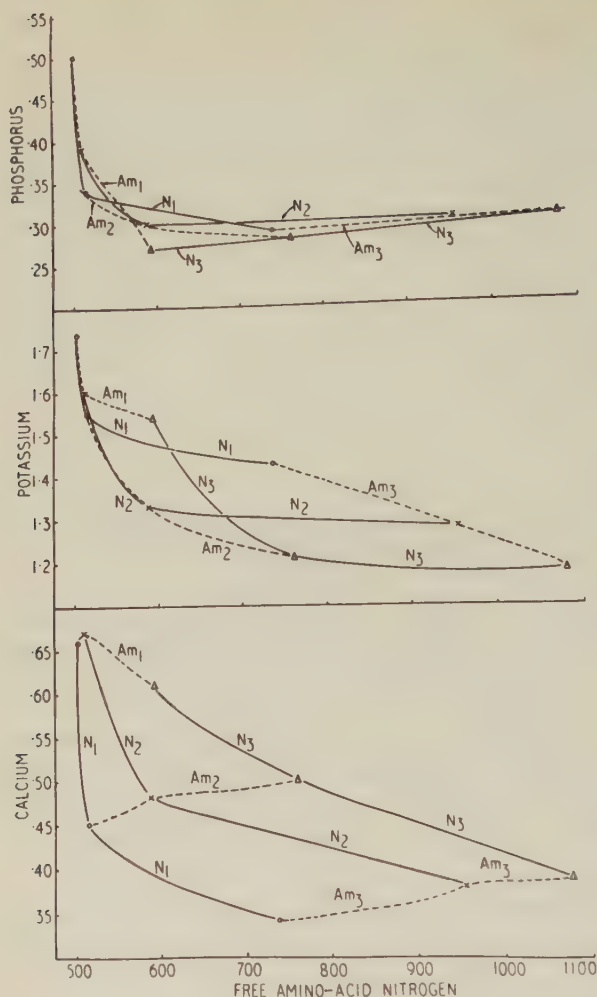


Figure 8. Relationship between free amino-acid nitrogen concentration of laminae and: phosphorus, potassium and calcium concentrations. (Amino -N expressed as p.p.m. of dry matter and P, K and Ca as % of dry laminae). Overall means for May, July, September and November values. Continuous lines denote effect of increasing ammonium supply; broken lines denote effect of increasing nitrate supply.

Magnesium. (Table 7). — Increased supplies of ammonium nitrogen decreased (particularly at the N_3 level) the concentration of magnesium in the laminae, whereas increased nitrate supply produced an overall increase in magnesium concentration. The effect of nitrate addition varied according to calcium level, a marked increase of magnesium concentration occurring only at the Ca_1 level; this $N \times Ca$ interaction effect was highly significant.

Increased calcium supply markedly decreased the magnesium concentration of the laminae.

Calcium. (Table 7). — As for magnesium, but more markedly so, the calcium concentration of the laminae decreased with increased supplies of

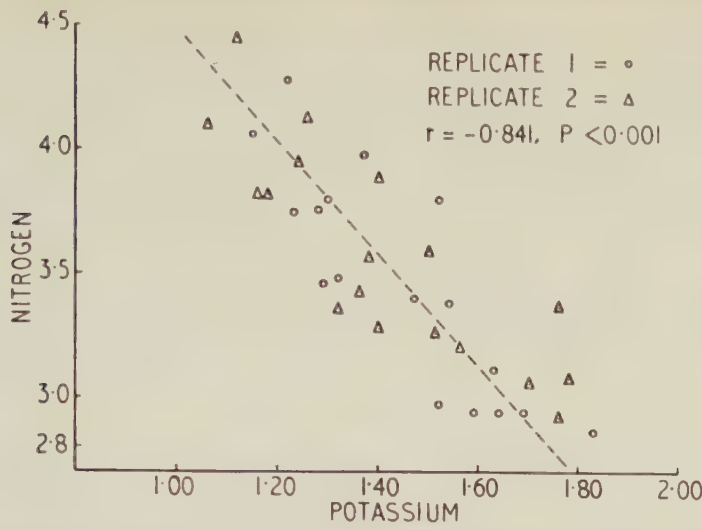


Figure 9. Variation of potassium concentration of laminae with total nitrogen concentration. (Potassium and nitrogen expressed as % of dry matter). Means of May, July, September and November values.

Table 7. Magnesium and calcium concentrations in laminae. May, July, September and November samplings. Mean values — expressed as % of dry laminae,

Level	Am ₁	Am ₂	Am ₃	Ca ₁	Ca ₂	Mean
Magnesium						
N ₁22	.22	.20	.23	.20	.22
N ₂23	.25	.22	.26	.20	.23
		(± .009)		(± .007)		(± .005)
N ₃26	.26	.21	.28	.20	.24
Ca ₁28	.27	.23			.26
Ca ₂20	.22	.18			(± .004) .20
Mean	.24	.24	.21	.26	.20	.23
		(± .005)		(± .004)		
Calcium						
N ₁66	.45	.34	.36	.62	.49
N ₂67	.48	.38	.32	.70	.51
		(± .022)		(± .018)		(± .013)
N ₃61	.50	.39	.30	.70	.50
Ca ₁44	.31	.24			.33
Ca ₂86	.65	.50			(± .011) .67
Mean	.65	.48	.37	.33	.67	.50
		(± .013)		(± .011)		

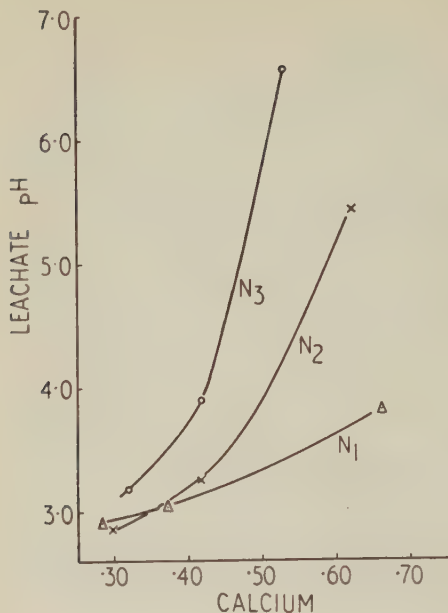


Figure 10. Relationship between calcium concentration of laminae and leachate pH. (Calcium expressed as ‰ of dry matter). Overall means for September and November values.

ammonium nitrogen. Nitrate addition generally had the reverse effect at the Am_2 and Am_3 levels but produced a decrease at the Am_1 level. This $Am \times N$ interaction effect was significant but the overall effect of nitrate addition failed to achieve significance.

Increased calcium supply increased the calcium concentration of the laminae; the effect was greatest at the Am_1 and N_3 levels. Both the $Am \times Ca$ and $N \times Ca$ interaction effects were highly significant.

Whereas the phosphorus and potassium concentrations of the laminae tended to decrease as the free amino-nitrogen increased (whether caused by extra ammonium or nitrate supply) the calcium concentration showed a similar relationship only when the amino-nitrogen increase was due to extra ammonium supply (Figure 8). Nitrate addition was found to increase both free amino-nitrogen and the calcium concentrations at the Am_2 and Am_3 levels. Thus the amino-nitrogen fraction was not related as closely to calcium concentration as it was to potassium and phosphorus concentrations and variation in amino-nitrogen was not always related to calcium concentration in the same manner.

It was noteworthy that the calcium concentration of the laminae bore some relationship to the pH of the medium surrounding the roots (Figure 10). Thus the application of ammonium nitrogen at any one N level reduced both the pH of the leachate and the calcium concentration of the laminae.

Discussion

The results revealed that application of ammonium nitrogen may produce some effects which are similar to those of nitrate nitrogen (for example, effects on the concentrations of nitrogen, potassium, and phosphorus found in the laminae) and some which are dissimilar (effects on leachate pH, magnesium and calcium concentrations in the laminae).

Where the effect of nitrogen application varied according to the ionic form in which it was applied, this variation may have been partially explicable in terms of the more rapid absorption of the ammonium ion in relation to nitrate and other ions and the consequent differential change in pH of the nutrient medium surrounding the roots (see »Leachate Composition«). These changes may have ultimately affected the concentration of the minerals in the leaves (Figure 10). An alternative explanation is suggested by work carried out by Gilbert, Shear, and Gropp (1951). They showed, for the tung tree, that the formation of oxalic acid was directly related to the nitrate supply; an increased proportion of ammonium to nitrate in the nutrient decreased the amount of oxalic acid produced. On this basis, the decreased uptake of calcium due to increased ammonium supply (Table 7) might be the result of the lowered oxalate content of the plant and the smaller quantity of insoluble calcium oxalate needed to be formed. Such a simple relationship could also be extended to explain the opposing effects of ammonium and nitrate on various other constituents whose accumulation was related to the amount of calcium absorbed. Militating against this possible explanation is a report made by Olsen (1939) who showed that, although ammonium ions inhibited the absorption of calcium, spinach plants fed with ammonium nitrogen contained more oxalic acid than others grown in a nitrate medium. However, if it can be shown that *Hevea brasiliensis* reacts in the same manner as the tung tree, and produces more oxalic acid as the nitrate-ammonium supply ratio increases, it may prove possible to resolve the differential effect of these two sources on that basis, particularly in view of the recent findings of Finkle and Arnon (1954). These workers demonstrated the presence of an oxalic acid oxidase, in the cytoplasmic particles of the sugar beet leaf; this enzyme oxidatively decomposed oxalate. Thus there is the possibility that the oxalic acid found in plants is not an inert end-product of metabolism but may be further utilised.

Where the effect of nitrogen application did not vary according to the form in which nitrogen was supplied it produced an overall response which was dependent solely on an increased acquisition of nitrogen; this was, presumably, soon metabolised and elaborated into compounds which affected the laminar concentrations of rubber, chlorophyll, and potassium (Figures 3, 4, 9). Significant but negative linear interaction effects between ammonium and nitrate levels were recorded for girth, dry weight and phosphorus concentration (Tables 1, 6). Thus, the effect of ammonium application on dry weight, for example, was less at the N_3 than at the N_1 level (Table 1). On the other hand, ammonium and nitrate levels interacted to produce a significant positive linear effect on ammonia- and amino-, nitrogen, con-

centrations in the laminae (Tables 4,5); this implied that the effect of either ammonium or nitrate addition on these concentrations increased with N or Am level, respectively.

The finding that the free amino-acid nitrogen fraction of the laminae increased as the concentrations of potassium and phosphorus decreased (Figure 8) was in agreement with reports of previous investigations (Wall, 1940, Sideris and Young, 1946, Cooil and Slaterry, 1948; Gregory, 1937, Steinberg, Bowling and McMurtrey, 1950). It appeared that these inter-relationships were largely independent of the form in which nitrogen was applied, unlike that observed for calcium in which the effect was only apparent when the free amino-acid nitrogen increase was caused by an increased supply of ammonium nitrogen (Figure 8).

Figure 7, provides evidence of the close relationships which existed between the total nitrogen concentration and the amino, amide and ammonia fractions in the laminae. The similarity in shape between the amino and amide curves indicated a probable interconvertibility between these fractions. The more linear appearance of the total nitrogen/nitrate curve suggested that nitrate concentration was not limited by any internal factor but depended more on the level of external supply of nitrogen. The remaining soluble nitrogen fractions were however influenced not only to the extent of their primary synthesis but also by various breakdown processes of more elaborate nitrogenous substances.

It seemed likely that at the higher levels of ammonium supply a detoxication mechanism, whereby excessive amounts of the ammonium ion were oxidised to nitrate, became operative. This would account for the increased nitrate concentration found in the laminae when the ammonium level (of the N₂ and N₃ nutrients) was increased from Am₂ to Am₃ (Table 4) as there was no evidence of corresponding nitrification taking place in the sand (Figure 2). A report of a similar detoxication mechanism in rice has been made by Malavolta (1954). An alternative explanation is to assume that ammonium was preferentially utilised in the presence of nitrate which would cause an accumulation of the latter in the laminae. These alternative explanations could be resolved if it were possible to grow *Hevea* in a sterile medium supplied with ammonium nitrogen only.

Figure 6 indicates that an increased nitrogen concentration in the laminae attributable to nitrate absorption invariably increased the girth measurement of the stem whereas nitrogen increases caused by extra ammonium supply did not always appreciably influence the girth. Thus nitrification rate and the balance of nitrate to ammonium ions in soil solution may, under field conditions, play a large part in the development of rubber trees of suitably large girth for commercial tapping purposes. This investigation has not disclosed the identity of the factor which is favourably influenced by nitrate (but not always by ammonium ion) addition and which promotes the girth increase.

As the nitrogen concentration of the laminae increased the rubber concentration decreased (Figure 3); this negative correlation achieved significance at the 5 % level. A similar reduction in the rubber concentration of bulked stems and petioles (not presented here) with increased ammonium and nitrate levels was also observed. The negative effect of nitrate (significant for laminae and for bulked stems and petioles) was usually more consistent than that of ammonium, but the difference in degree was not of sufficient magnitude to be important. Thus, as the nitrogen concentration of the laminae increased, the chlorophyll also increased but the rubber concentration decreased (Figures 3, 4). These results are at variance with those obtained from a previous investigation conducted on field grown plants; it was then found that the nitrogen concentration of the laminae was positively correlated with the rubber concentration of the petioles (Bolle-Jones and Ratnasingam, 1954). The range of laminar nitrogen concentration covered in the present investigation was much wider. This may imply that the present results possess a greater general validity but does not explain why increased nitrogen and chlorophyll concentrations in the laminae accompanied a decrease in rubber concentration. On *a priori* grounds it had been anticipated, wrongly, that a high chlorophyll concentration would enhance the amount of rubber present. Further investigation is necessary to establish with certainty the effect of nitrogen level on various organic constituents of the lamina which may be involved in the formation of rubber.

It has been noted (for *Hevea brasiliensis*) that, even under conditions of severe deprivation of calcium supply, diagnostic symptoms of deficiency may be absent although the growth of the plants and the laminar calcium concentration were much reduced (Bolle-Jones 1954 b). The present experiment confirmed that, despite the low level of calcium administered in the Ca_1 nutrients, visual indications of malnutrition were absent. Frequently, the general appearance and total dry weight per plant were similar for plants receiving different ratios of ammonium to nitrate supply (for example, $Am_1 N_2$ — 339 g: $Am_2 N_1$ — 351 g.) whereas the calcium concentrations of the laminae differed significantly ($Am_1 N_2$ — 0.65 % Ca; $Am_2 N_1$ — 0.40 % Ca). The remarkably low requirement for calcium (as reflected in the low calcium concentration at the $Am_3 Ca_1$ level, Table 7) coupled with the plant's tolerance of a low pH may represent an adaptation to strongly acid tropical soils and may explain the facile cultivation of the crop under Malayan conditions. At any one nitrate level the calcium concentration of the laminae decreased as the pH of the leachate solution decreased (Figure 10), as a result of increasing ammonium supply, but the total dry weight per plant did not usually decrease (Table 1). It is not known whether the low pH of the medium surrounding the roots directly reduced the absorption of

calcium or whether the rapid absorption of the ammonium ion hindered calcium uptake. The work of Arnon *et alii* (1942 a, b) suggests that a low pH may directly decrease calcium absorption; Steinberg (1949) has shown, for tobacco, that increased acidity increased the requirement for calcium if the same level of dry matter production was to be maintained. Olsen (1953) claimed that the pH value of the nutrient solution, *per se*, had no direct effect on ion uptake except below 3.5 and above 10.5 at which direct damage was caused. Nevertheless it is generally agreed that an increased supply of ammonium nitrogen will decrease the uptake of calcium (Olsen 1939; Hewitt 1952, p. 95); there can be little doubt on the basis of the present experiment that the requirement of Hevea for calcium seemed to decrease as the ammonium content of the nutrient increased.

Calcium addition did not induce many significant responses except in regard to the following laminar constituents; (positive) ammonia-nitrogen, calcium; (negative) chlorophyll, total nitrogen, amide-nitrogen, magnesium. It was interesting to note that calcium addition increased the ammonia-nitrogen concentration of the laminae but decreased that of amide-nitrogen. This might suggest that increased calcium concentration within the laminae caused a partial decomposition of the amides with the production of ammonia. Steward and Preston (1941) have also indicated that application of calcium (as the nitrate) appreciably increased the ammonia-nitrogen accumulated in potato discs.

From the standpoint of practical application the significant interaction effects between nitrate and calcium levels on magnesium and calcium contents were of importance (Tables 6, 7). At a low calcium level nitrate application markedly enhanced the magnesium concentration of the laminae and reduced that of calcium, but at the higher calcium level nitrate application did not affect magnesium but increased the concentration of calcium. Accordingly in the widely distributed and severely affected magnesium deficient areas of Malaya it may prove advantageous to apply nitrate (or ammonium fertilisers if nitrification conditions are favourable) in the absence of calcium containing fertilisers; this action should increase the concentration of magnesium within the laminae.

The outstanding information gained from this investigation from the standpoint of future sand culture experiments was the tolerance of Hevea to relatively high concentrations of the ammonium ion in the nutrient. The five milligram-equivalents of ammonium salt applied per litre at the Am_3 level represented a higher concentration than usually applied in recently developed »typical nutrient solutions» as quoted by Hewitt (1952). Very slight but not significant indications of toxicity, as measured in terms of girth and dry weight, were observed at the lower calcium level (Table 1). Extreme susceptibility of Hevea to iron chlorosis, and a relatively high requirement for iron in the nutrient to avoid this chlorosis, are commonly experienced

when the plant is supplied with nitrate nitrogen (Bolle-Jones 1954 a, b). The well marked and better response of chlorophyll production to ammonium application (as compared with nitrate) in this experiment and the complete absence of chlorosis at the higher ammonium levels indicates the desirability of using ammonium salts for all subsequent sand culture experiments with *Hevea*.

Summary

1. Seedlings of *Hevea brasiliensis* were grown in sand culture and were supplied with varying ratios of ammonium and nitrate nitrogen and a very low or normal level of calcium. The plants tolerated a high level of ammonium supply and the low level of calcium without any serious or permanent visual indication of malnutrition.

2. Ammonium or nitrate addition exercised a similar positive effect on dry weight of plant, and on the concentrations in the laminae of chlorophyll, ammonia-, nitrate-, amide-, free amino-acid and total-, nitrogen, and similar negative effects on the laminar concentrations of phosphorus and potassium. For the chlorophyll, ammonia-, nitrate-, amide-, free amino- and total-, nitrogen, and potassium concentrations the overall response to ammonium was more marked than to nitrate.

3. Ammonium and nitrate exerted differential effects on the magnesium and calcium concentrations of the laminae and on the pH of the medium surrounding the roots. Ammonium addition decreased the magnesium and calcium laminar concentrations and of the (leachate) pH, while nitrate addition increased the laminar concentration of magnesium for the low calcium plants, the laminar concentration of calcium for the high calcium plants and the (leachate) pH.

4. The inclusion of ammonium salts as a source of nitrogen supply decreased the requirement for calcium and did not always increase the stem girth measurement of the plants as nitrate addition invariably did.

5. As the potassium and phosphorus concentrations of the laminae decreased, the free amino-acid nitrogen present increased. A similar increase was obtained for a decreased laminar concentration of calcium but only when that decrease was caused by extra ammonium supply.

6. Increased calcium supply decreased the chlorophyll and amide-nitrogen concentrations within the laminae but increased that of ammonia-nitrogen at the lower levels of ammonium supply.

7. The ability of *Hevea brasiliensis* to withstand extremely low pH values and relatively high ammonium concentrations, and the requirement for a

very small supply of calcium, may be a partial explanation of the successful cultivation of the crop under tropical, strongly acid, soil conditions.

I thank Mr. Chin Tet Tsoy for his care and maintenance of the growing plants and Mr. K. Ratnasingam for his supervision of the chemical analyses.

References

- Arnon, D. I., Frazke, W. E. & Johnson, C. M.: Hydrogen ion concentration in relation to absorption of inorganic nutrients by higher plants. — *Plant Physiol.* 17: 515. 1942, a.
- & Johnson, C. M.: Influence of hydrogen ion concentration on the growth of higher plants under controlled conditions. — *Plant Physiol.* 17: 525. 1942, b.
- Benedict, H. M.: Factors affecting the accumulation of rubber in seedling guayule plants. — *Bot. Gaz.* 112: 86. 1950.
- Bolle-Jones, E. W.: Nutrition of *Hevea brasiliensis*. I. Experimental methods. *J. Rubb. Res. Inst.* — *Malaya* 14: 183. 1954, a.
- Nutrition of *Hevea brasiliensis*. II. Effect of nutrient deficiencies on growth, chlorophyll, rubber, and mineral contents of Tjirandji I seedlings. — *J. Rubb. Res. Inst. Malaya.* 14: 209. 1954, b.
- & Ratnasingam, K.: Nutrition of *Hevea brasiliensis*. IV. Interclonal and seasonal variation in composition of leaves. — *J. Rubb. Res. Inst. Malaya,* 14: 257. 1954.
- Bonner, J.: Effect of varying nutritional treatments on growth and rubber accumulation in guayule. — *Bot. Gaz.* 105: 352. 1944.
- Burström, H.: The colorimetric determination of nitrate in plant materials. — *Svensk Kem. Tidskr.* 54: 139. 1942.
- Cooil, B. J. & Slattery, M. C.: Effect of potassium deficiency and excess upon certain carbohydrate and nitrogenous constituents in guayule. — *Plant Physiol.* 23: 425. 1948.
- Finkle, B. J. & Arnon, D. I.: Metabolism of isolated cellular particles from photosynthetic tissues II. Oxidative decarboxylation of oxalic acid. — *Physiol. Plant.* 7: 614, 1954.
- Gilbert, S. G., Shear, C. B. & Gropp, C. M.: The effects of the form of nitrogen and the amount of base supply on the organic acids of tung leaves. — *Plant Physiol.* 26: 750. 1951.
- Graham, J.: Manurial experiments on *Hevea*. — *Arch. Rubbercult.* 8: 501. 1924.
- Manurial experiments on *Hevea*. II. — *Arch. Rubbercult.* 11: 465. 1927.
- Manurial experiments on *Hevea*. III. — *Arch. Rubbercult.* 14: 345. 1930.
- Gregory, F. G.: Mineral nutrition of plants. — *Ann. Rev. Biochem.* 6: 557. 1937.
- Haines, W. B.: Manuring *Hevea*. IV. Conspectus of experimental improvements achieved in mature stands at the end of ten years with a special note on seed production. — *Emp. J. Exp. Agric.* 14: 182. 1946.
- & Guest, E.: Recent experiments on manuring *Hevea* and their bearing on estate practice. — *Emp. J. Exp. Agric.* 4: 300. 1936.
- Hewitt, E. J.: Sand and water culture methods used in the study of plant nutrition. — *Communication* 22. *Tech. Commun. Bur. Hort. E. Malling.* 1952.
- Malavolta, E.: Studies on the nitrogenous nutrition of rice. — *Plant Physiol.* 29: 98. 1954.
- Meeks, J. W., Crook, R. V., Pardo, C. E. & Clark, F. E.: Determining rubber hydrocarbon in rubber-bearing plants. — *Anal. Chem.* 25: 1535. 1953.
- Okanenko, H. S. & Bershtein, B. I.: Characteristics of the oxidation — reduction processes in kok-saghyz on feeding with different sources of nitrogen. — *Dopovidi Akad. Nauk. Ukrain R.S.R.* p. 317. 1950. (C.A. 46: 6855).

- Olsen, C.: Absorption of calcium and formation of oxalic acid in higher green plants. — *Compt. rend. Lab. Carlsberg, Ser. Chim.*, 23: 101. 1939.
- The significance of concentration for the rate of ion absorption by higher plants in water culture. IV. The influence of hydrogen ion concentration. — *Physiol. Plant.* 6: 848. 1953.
- Pinevic, L. M.: The effect of different sources of nitrogen nutrition on the accumulation of rubber in koksaghyz. — *Doklady Akad. Nauk. S.S.S.R.* 82: 485. 1952. (*Hort. A.* 23: 139).
- Sideris, C. P. & Young, H. Y.: Effects of potassium on the nitrogenous constituents of *Ananas Comosus* (L) Merr. — *Plant Physiol.* 21: 218. 1946.
- Sobel, A. E., Hirschman, A. & Besman, L.: A convenient microtitration method for the estimation of amino-acids. — *J. Biol. Chem.* 161: 99. 1945.
- Steinberg, R. A.: Effect of initial acidity on calcium and magnesium requirements of tobacco in asptic culture. — *J. Agric. Res.* 75: 251. 1947.
- Bowling, J. D. & McMurtrey, J. E.: Accumulation of free amino acids as a chemical basis for morphological symptoms in tobacco manifesting frenching and mineral deficiency symptoms. — *Plant Physiol.* 25: 279. 1950.
- Steward, F. C. & Preston, C.: The effect of salt concentration upon the metabolism of potato discs and the contrasted effect of potassium and calcium salts which have a common ion. — *Plant Physiol.* 16: 85. 1941.
- Varner, J. E., Bulen, W. A., Vanecko, S. & Burrell, R. C.: Determination of ammonium, amide, nitrite and nitrate nitrogen in plant extracts. *Anal. — Chem.* 25: 1528. 1953.
- Vollema, J. S.: Bemestingsproeven bij Hevea in West-Java. — *Arch. Rubbercult.* 15: 481. 1931.
- Wall, M. E.: The role of potassium in plants: III. Nitrogen and carbohydrate metabolism in potassium-deficient plants supplied with either nitrate or ammonium nitrogen. — *Soil Sci.* 49: 393. 1940.

Protochlorophyll Formation and Greening in Etiolated Barley Leaves

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Introduction

In works by Koski (6), Koski and Smith (7), Koski et al. (8), Smith and Benitez (22), and Virgin (25), clear evidence has been presented supporting the hypothesis that protochlorophyll is the precursor of chlorophyll a. At least this is the case in early stages of chlorophyll a formation in an etiolated plant. In this paper it will be shown that in the later stages of the process other chains of reactions leading to the formation of chlorophyll a are indicated.

Three distinct stages are demonstrable during chlorophyll formation when an etiolated plant is subjected to light. First, the small amounts of protochlorophyll always present in etiolated plants undergo rapid photochemical transformation to chlorophyll a. This transformation takes place almost immediately when the plant is exposed to light. Since the amount of protochlorophyll present in an etiolated leaf is very small, so also is the amount of chlorophyll a formed from it.

Second, additional chlorophyll a is formed. This so-called greening process is much slower than the first step. The concentration of chlorophyll a in a normal leaf is several thousand times that of chlorophyll a formed from the protochlorophyll initially present (cf. Smith, 17). Therefore, proto-

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chlorophyll, if it is the precursor of chlorophyll *a*, must be formed continuously while an etiolated plant is being illuminated and chlorophyll *a* is being produced. Since very little protochlorophyll can be detected in a green leaf in light, its supposed formation must be at a rate very slow in comparison to the rate of its protochemical conversion.

The third stage is the formation of chlorophyll *b*. The first traces of chlorophyll *b* appear after about one hour of continuous illumination (Blaauw-Jansen et al., 1 and Smith, 20). The precursor of chlorophyll *b* is not known. Seybold (15) suggested such a precursor from studies of the protochlorophyll in the inner seed coats of squash but his findings have not got any support by later authors, cf. Smith and Young (23).

Most plants need light to form chlorophyll *a*. Exceptions to this are seedlings of certain gymnosperms and others. In this case photochemical reactions seem to have been replaced by an enzymatic process. Also here protochlorophyll is reported in small amounts but it is not known whether the formation of chlorophyll *a* in such plants goes through protochlorophyll. (Cf. Smith and Young, 23). Some parts of certain plants, for example the seed coat of pumpkin seeds are rich in a form of protochlorophyll which can not be converted (Noack and Kiessling, 10, 11).

As already mentioned, a certain amount of protochlorophyll is present in most plants which have developed in complete darkness. The concentration level may vary according to the genetic constitution (Koski, 5). Little is known regarding possible restoration of this level in an illuminated etiolated leaf which is replaced in darkness or about the importance of the temperature factor in such restoration.

Schnarfnagel (14) found photographic indication of protochlorophyll bands after an illuminated leaf of *Zea Mays* had remained in darkness for 10—20 hours. No quantitative measurements were made. Rudolph (13) confirmed Schnarfnagel's observations, and was also able to detect the first reappearance of protochlorophyll after the plant had been in darkness for about eight hours.

Smith and Young (23) report a reappearance of protochlorophyll in barley leaves which had been kept in darkness after a short irradiation. If an etiolated leaf is illuminated for about five minutes, then placed in darkness for 55 minutes, and this process is repeated over a period of several hours, the chlorophyll content of the leaf is increased (Smith, 20). On the other hand, if this process is carried on at low temperatures, no further chlorophyll is formed. This indicates that protochlorophyll is not formed at low temperatures. In this paper the relationship between rate of reappearance of protochlorophyll in darkness at different temperatures and the formation of chlorophyll *a* and *b* in light has been studied.

Techniques

Plant material

The experiments were made with etiolated leaves of the strain of barley used by Smith and Benitez (22). The treatment and cultivation were in accordance with earlier described experiments with protochlorophyll (Virgin, 25).

Determination of pigment concentrations

Determination of the pigment concentrations was based on fluorescence measurements. The apparatus used was described in earlier papers (French and Young, 3; Virgin, 24, 25).

For a pigment determination three leaves were cut into small pieces. These were ground in a glass mortar with 4 ml. of acetone. This was done in very dim green light. The acetone extract was transferred to a Beckman cell for fluorescence measurement.

Control measurements showed filtration of the extract to be unnecessary since filtered and unfiltered solutions did not differ appreciably in fluorescence yield. The glass cell containing the extract was covered with aluminum foil which had an opening the same size as the entrance slit of the analyzing monochromator. This was done to be sure that in each measurement the fluorescent light would follow the same path in the solution, and thus the fluorescent radiation was taken from the same solid angle in each case.

In determining fluorescence of protochlorophyll and chlorophyll a, incident light of 436 m μ from a mercury lamp was used, isolated by means of Corning filters No. 3389, 5113, and 4305. For determination of chlorophyll b fluorescence, the exciting radiation was isolated from the light of a 1000 watt incandescent lamp by means of a combination of Corning filters No. 3389, 5030, 4303 and 4305. This radiation band had a maximum intensity at 456 m μ .

Determinations of the concentration of protochlorophyll and chlorophyll a were done according to the procedure earlier described (Virgin, 25). Chlorophyll b was determined in the presence of chlorophyll a (protochlorophyll was never present, see below). The determination of the individual pigments is complicated by the proximity of their main fluorescence bands. Chlorophyll b dissolved in acetone has its main fluorescence peak at 653 m μ , and chlorophyll a at 668 m μ . If an extract containing both pigments is illuminated with light absorbed by both pigments and one pigment is present in small amount, its fluorescence peak will appear only as a shoulder on the fluorescence curve observed. But by adding fluorescence curves of the individual pigments in known ratios the observed curve can be reproduced and the

relative proportion of the two pigments calculated (cf. French and Young, 3). The labor involved in such an analysis can be greatly lessened by using the curve analyzer developed by French et al. (4).

It should be pointed out that in this paper all measurements of protochlorophyll are made in relation to chlorophylla concentration and conversely. The experiments were designed so that either one pigment or the other should, as far as is known, remain constant under the experimental conditions. In all cases one of the pigments was used as an internal standard and the results report the variation of the other assuming a constant concentration of the pigment used as a standard.

Experimental

Formation of protochlorophyll in previously illuminated, etiolated leaves

Etiolated barley leaves were irradiated for one hour with two fluorescent lamps, (Mazda daylight, 15 watt), 5 cm. apart and 27 cm. from the leaves. The intensity of the light, measured with a Weston meter, was 200 foot candles. During illumination the leaves were evenly distributed over moist filter paper in a dish covered with a glass plate. Care was taken that the leaves did not cover each other.

After illumination the leaves were transferred to darkness where they were kept for different periods of time and under five different temperatures: 0, 8, 15, 22, and 30° C. The period in darkness varied from 30 minutes to 12 hours except in one series where the regeneration of protochlorophyll was also determined after 30 hours in darkness.

During illumination practically all protochlorophyll, initially present, is converted into chlorophylla. The quantity of chlorophyll would thus be about the same in all samples when returned to the dark and would remain so since there is no evidence for a change in the content of chlorophylla during the storage in darkness (Figure 1). The level of chlorophylla can therefore be used as a standard for calculation of the amount of protochlorophyll.

As shown in Figure 1, new protochlorophyll is formed in darkness. Figure 2 illustrates how this formation of protochlorophyll takes place at different temperatures. It can be seen that formation of new protochlorophyll begins more or less immediately after the plants are transferred to darkness. As has been mentioned by Lubimenko and Hubbenet (9), the formation of protochlorophyll is completed after about 7—10 hours. Thereafter the level stays constant. It is of interest that this end level is different

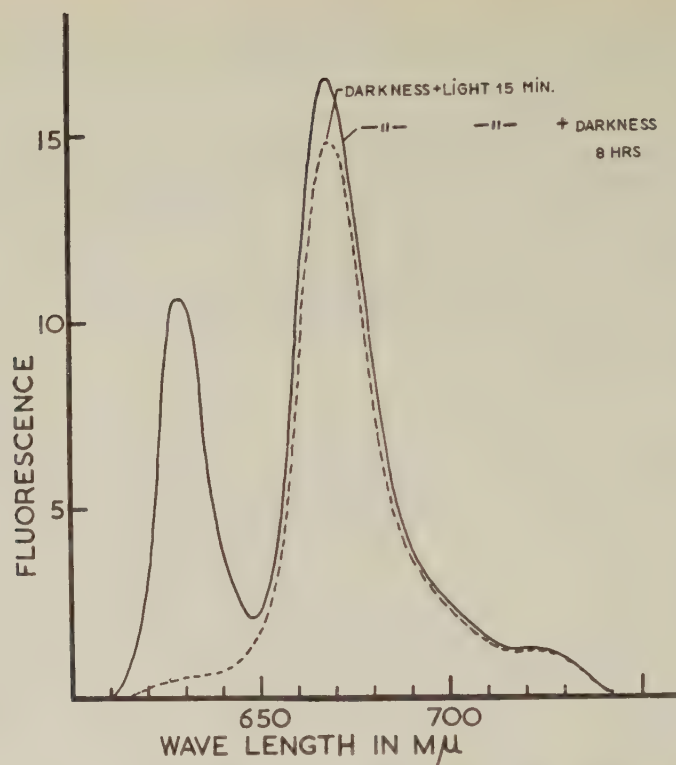


Figure 1. *Fluorescence spectra of barley leaves.* Broken line: dark grown leaves, illuminated for 15 minutes. Solid line: the same as before, but after an additional stay in darkness for eight hours. The spectra are from acetone extracts of seven leaves. The two peaks derive from protochlorophyll and chlorophyll a with their maxima of fluorescence intensity at 628 mμ and 668 mμ respectively.

for different temperatures, and is not only due to a different rate of protochlorophyll formation. For example, storage in darkness for about 30 hours at 8° C does not increase the value beyond that noted after 10 hours. At 0° C no formation of protochlorophyll takes place, which is in agreement with facts known about protochlorophyll and chlorophyll a formation at low temperatures (Smith, 18, 19).

Increasing temperatures give increasing rates of formation as well as greater end values. These changes are larger for temperatures between 0 and 15° C than between 15 and 30° C. In fact, an increase in temperature from 15° C to 30° C does not change the rate of formation to any large extent. Whereas Q_{10} for lower temperatures is around 2, it is little more than 1 for a rise in temperature between 22 and 30° C.

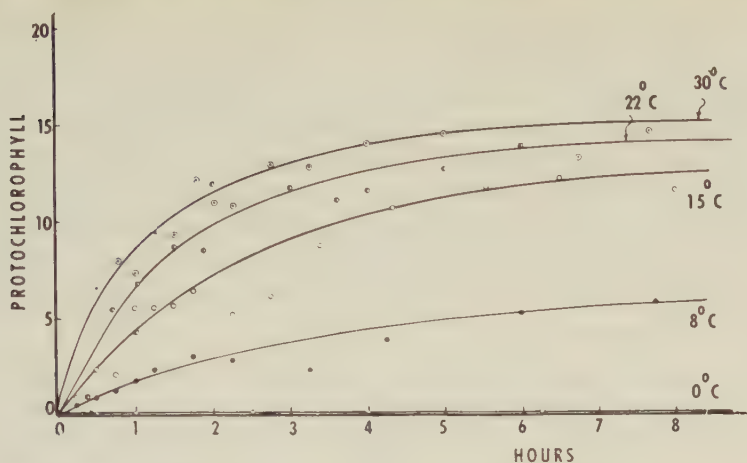


Figure 2. Formation in darkness of protochlorophyll at different temperatures in etiolated and illuminated barley leaves. The units along the y-axes in Figures 2–5 are relative but are all comparable, as one unit of protochlorophyll corresponds to one unit of chlorophyll. Consideration is taken to the difference between fluorescence yield of protochlorophyll and chlorophyll a.

The process of greening

a) *Influence of temperature.* — An etiolated plant which is exposed to light will soon assume a pale green color due to chlorophyll formation. To reach maximum chlorophyll concentration the illumination has to last for about two days.

So far as is known, the action spectrum for greening during the first hours of illumination is the same as that for protochlorophyll—chlorophyll a conversion. This conclusion is warranted by the following facts. The same type of spectrum is obtained when the actual percentage of protochlorophyll converted to chlorophyll a is measured in short experiments during which no further protochlorophyll is formed (Koski et al., 8) as when measurements of the action spectrum are based on calculations of light energy necessary to form an appreciable amount of chlorophyll during five hours illumination (Frank, 2). After this time more chlorophyll is formed than can be accounted for by the initial amount of protochlorophyll present.

The course of the greening process with time has been followed by several authors. After the initial transformation the general trend is a rather slow accumulation of chlorophyll a at the beginning of greening. After 10 hours the accumulation is much accelerated. Therefore, if chlorophyll a is formed from protochlorophyll, the protochlorophyll formation must in some way

be influenced by light either through direct action or through the (photo-synthetic?) products formed.

As shown in the previous section, the rate of formation of protochlorophyll in darkness is different for different temperatures. If protochlorophyll is the precursor of chlorophyll *a* the rate of chlorophyll *a* accumulation should follow the initial rate of protochlorophyll formation when light intensities just strong enough for the conversion are used. Since the rates of protochlorophyll formation vary with temperature the rates of chlorophyll *a* accumulation should vary in like manner with temperature. Experiments to test this were performed.

Barley leaves grown in the dark were illuminated at different temperatures at the same intensity of light from fluorescent tubes as already described for protochlorophyll transformation. During illumination the leaves were placed on moist filter paper in a culture flask, »Kolle» type, provided with a rubber stopper. The flask was immersed in water kept at a constant temperature. Glass tubes through the stopper permitted circulation of air inside the flask.

When samples were taken, the flask was removed from the water bath for a few minutes. This did not appreciably change the temperature as measured by a small thermometer inside the flask. The largest temperature fluctuation was 1° C for two minutes. This change is of no importance, as the change in pigment concentration, after the first rapid conversion of protochlorophyll, is a slow process.

After different periods of illumination leaves were taken from the chamber and transferred to complete darkness where they were kept for eight hours at 22° C. The pigment concentration was then determined. During this time in darkness protochlorophyll will be formed and reach a steady state as described in the previous section. It was shown that the different amounts of chlorophyll *a* formed in the leaves do not affect the steady state level of protochlorophyll after eight hours in darkness, at least not after an illumination period of 30 minutes to four hours. This means the protochlorophyll level can be used as a standard for calculation of the relative amounts of chlorophyll *a* which are formed. Results of these experiments are shown in Figure 3.

If we first consider the formation of chlorophyll during the initial hours of illumination, it is evident that the formation rate follows a more or less straight line and differs for different temperatures. From a comparison of the slopes of these lines to the slopes of the curves for protochlorophyll formation at the beginning of the process, the conclusion can be drawn that they show fairly good agreement for 30 and 22° C but not for lower temperatures (Figure 4). Here light-destruction of chlorophyll *a* (or protochlorophyll?) is noted which is especially evident at 8° C where, during part of the illumination, the destruction predominates the formation.

Such anomalies in curves for chlorophyll *a* formation have been observed

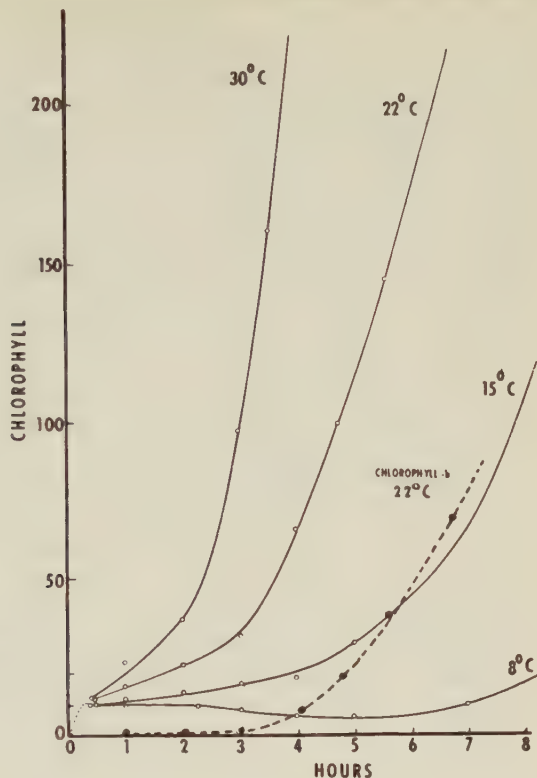


Figure 3. Formation of chlorophyll at different temperatures in etiolated and illuminated barley leaves. Solid lines: chlorophyll a. Broken line: chlorophyll b at 22° C.

by many authors (Blaauw-Jansen et al., 1; Smith, 19 a.o.). It is obvious that we must always expect partial destruction of the pigments during illumination as associated with the photooxidation of the pigments in vitro and in vivo (cf. Rabinowitch, 12; p. 537). The effect will be particularly obvious at low temperatures where destruction of pigment predominates over formation. It is probable, therefore, that the peculiar shape of the curves of Blaauw et al. (1) is due to a chlorophyll destruction which shows up clearly when chlorophylla formation is low. As the formation increases above the rate of destruction the curve begins to rise again. When the destruction is taken into account the formation of chlorophylla probably follows the formation of protochlorophyll during the first hours of illumination. If this is true, the destruction, however, seems to be greatest at low temperatures. After a few hours of illumination some new factor enters and the rate of formation increases considerably. The beginning of this increase is dependent on temperature as it occurs earlier at higher temperature.

In a normal leaf the ratio of chlorophyll a to chlorophyll b is about 3 to 1. During the initial stage of the greening of an etiolated leaf only chlorophyll a

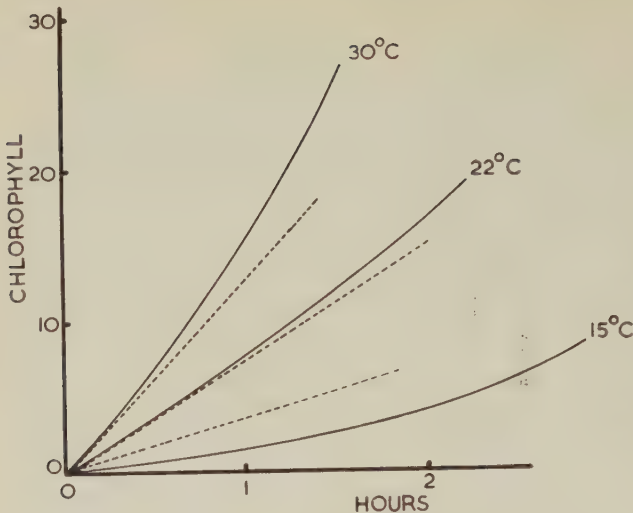


Figure 4. Formation of chlorophyll *a* at different temperatures as compared with the rate of formation of protochlorophyll in darkness at the very beginning of the formation. Solid lines: chlorophyll *a*. Broken lines: protochlorophyll. The Figure is derived from data from Figures 3 and 4.

can be detected. At least it has not been possible with absorption spectrophotometry to trace chlorophyll *b* earlier than about one hour after the beginning of illumination (Blaauw-Jensen et al., 1 and Smith, 20). It would therefore be of interest if the sudden change in the rate of chlorophyll *a* formation as seen in Figure 3 has something to do with the appearance of chlorophyll *b*.

Detection of the first appearance of chlorophyll *b* was performed in the following way. Etiolated barley leaves were illuminated as mentioned above. Instead of keeping them in darkness for eight hours after illumination, they were extracted immediately with acetone and the extract examined for fluorescence. In this way no protochlorophyll was present to complicate the shape of the fluorescence spectrum obtained. From Figure 3 it is evident that the first traces of chlorophyll *b* appear after about one hour of illumination with light intensity of 200 foot candles. Thus, even by using this very sensitive method, chlorophyll *b* can not be traced earlier than approximately one hour after the beginning of the illumination. This means that the first traces of chlorophyll *b* appear in the plant before the sudden increase in chlorophyll *a* formation sets in.

Further analysis of the formation of chlorophyll *b* during continued illumination shows the rate of formation of this pigment in illuminated etiolated plants follows about the same trend as the rate of formation of chlorophyll *a*. It seems probable that two independent processes are involved and that both are affected by the same factor at the point of the sudden increase in formation rate.

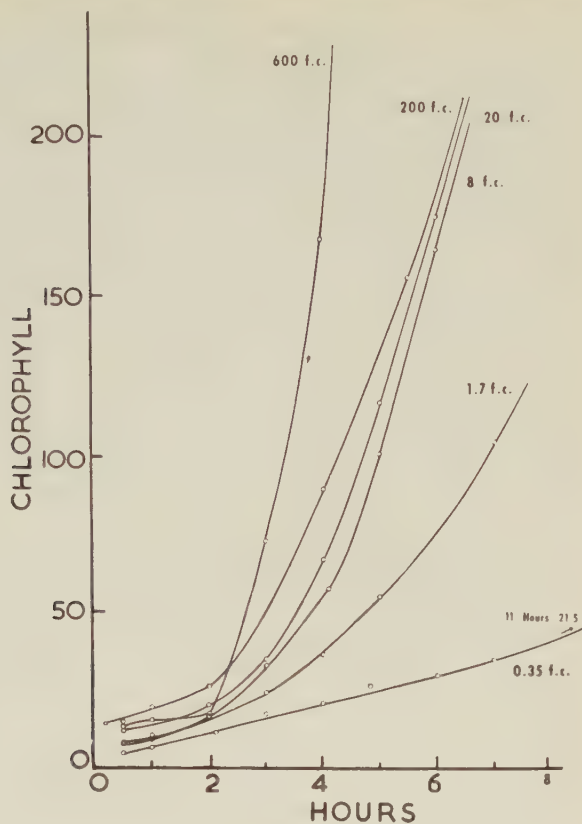


Figure 5. Formation of chlorophyll *a* at different light intensities in etiolated and illuminated barley leaves. Temperature: 22° C.

b) *Influence of light intensity.* — In the experiments so far reported in this paper, the intensity of the light was kept constant. If instead the temperature is kept constant (22° C) and the intensity of light is varied we get the curves shown in Figure 5. It was shown in Figure 3 that a process sets in after about three hours of illumination which results in accelerated formation of chlorophylls *a* and *b*. The curves in Figure 5 show that this reaction is dependent upon the light intensity.

For about the first three hours of illumination the rate of formation of chlorophyll *a* tends to follow the rate of protochlorophyll formation. The light intensity has no marked influence if it is above that which will convert the protochlorophyll as fast as it is formed.

The difference in position of the curves can be accounted for by considering that at any intensity of light an equilibrium is soon established between the protochlorophyll formed and the protochlorophyll converted into chlorophyll *a* per unit time.

At high light intensities most of the protochlorophyll present at the beginning of the illumination will be converted and consequently only small amounts will be present at equilibrium. With low light intensities larger amounts of protochlorophyll will be present continually due to slower conversion. Thus the position of the curve with respect to the y-axis (concentration of chlorophyll *a*) at the beginning of illumination will be determined by the percentage of protochlorophyll converted prior to attainment of equilibrium. After illumination with medium intensities for about three hours an acceleration in chlorophyll *a* formation sets in. There does not seem to be a linear relationship between light intensity and chlorophyll formation, however. At low intensities an increase in light gives rise to an acceleration in chlorophyll formation. Within the range of 8—200 foot candles the intensity does not seem to affect the chlorophyll formation to any notable extent. Above 200 foot candles the results indicate a further increase in pigment formation. The lower chlorophyll production at 600 foot candles than in the other series during the first two hours of illumination can be ascribed to the strong pigment destruction at these light intensities. This is clearly evident when the rate of pigment formation is low, but less evident when the acceleration of pigment formation sets in — after about 2—3 hours of illumination.

At a very low light intensity (0.35 foot candles) the formation of chlorophyll *a* approximately follows a straight line during the first eight hours of illumination. This indicates that the formation results from conversion of protochlorophyll which itself is formed at a constant rate. After about 11 hours of illumination even this curve for chlorophyll formation shows a more rapid rise.

It is evident from these experiments that an increase in light intensity causes the course of formation of chlorophyll *a* after several hours of illumination to differ from the course at the beginning. The shape of the curves with the onset of the sudden increase in rate of pigment formation gives the impression that the process may be autocatalytic. If that is the case, chlorophyll *a* itself can not be the catalyst since the curve for 0.35 foot candles follows a straight line even after the concentration reaches the level where the sudden increase appears at higher intensities. If it is true that all chlorophyll *a* is formed via protochlorophyll, it would seem that the rate of the formation of this precursor is influenced in some way by the light intensity, either directly or indirectly via some other substance.

Discussion

The experimental data presented in this paper give strong support to the hypothesis that at the start of the greening process chlorophyll *a* is formed in light at the same rate as protochlorophyll is formed in darkness. Due to the very rapid photochemical conversion of protochlorophyll into chlorophyll *a* at »normal« light intensities, the rate of formation of chlorophyll will be completely determined by the protochlorophyll formation.

Earlier studies of chlorophyll formation at different temperatures (Lubimenko and Hubbenet, 9) have also shown that the formation of chlorophyll *a* increases about 10-fold for a temperature rise from 5 to 15° C, but only 1.1—1.2 times for a rise from 18 to 28° C. As shown in this paper the formation of protochlorophyll in darkness at different temperatures has about the same temperature dependence as the chlorophyll *a* formation reported by these authors.

The fact that the constant rate of formation does not last for more than about one hour of illumination with medium intensities, but is followed by a sudden increase in the formation, indicates that light has an effect on protochlorophyll formation. However, the possibility of quite another mechanism of chlorophyll *a* formation in this case can not be excluded. As already mentioned, Frank's (2) experiments have shown that even after illumination for more than five hours an action spectrum for the formation of chlorophyll *a* is noted which closely resembles the absorption spectrum for protochlorophyll. However, it can be assumed that the light intensities in these experiments by Frank were too low to cause the sudden acceleration in chlorophyll *a* formation, but allowed the formation to follow a straight line, as with 0.35 foot candles in Figure 5. Therefore it is uncertain whether the action spectrum for the later part of the greening process is the same as for the first where it is proved that the process occurs via protochlorophyll.

The origin of chlorophyll *b* is still obscure. It seems to be formed quite independently of chlorophyll *a*. The linear relationship between chlorophyll *a* and chlorophyll *b* seems to be more of an accidental occurrence than something essential to the plant. This conclusion can also be drawn from the fact that the ratio of the pigments can be altered by changing the conditions of illumination (Seybold and Eagle, 16). In addition it is known that chlorophyll *b* is not necessary for photosynthesis (Smith, 21) but probably acts as an auxiliary pigment in absorbing light energy.

Summary

The rate of formation in darkness of protochlorophyll in etiolated leaves of barley has been studied at different temperatures and has been compared with the formation of chlorophyll *a* in light. The concentration of the pigments have been determined by means of the intensity of their fluorescence.

The main results are:

1. The rate of protochlorophyll formation in darkness as well as the end level vary with temperature. The increase in rate is much more rapid for a rise in temperature within the interval 0°C to 15°C than between 15°C and 30°C . These changes in rate are accompanied by the same relative differences in the end levels.
2. The rate of chlorophyll *a* formation in light follows the rate of protochlorophyll formation in darkness during the first two hours of illumination with medium light intensities.
3. After about two hours of illumination an acceleration sets in in the formation of chlorophyll *a*. Whether this acceleration is accompanied by a corresponding acceleration of the protochlorophyll formation has not been established.
4. The first traces of chlorophyll *b* appear after about one hour after the beginning of illumination. This appearance does not seem to have anything to do with the aforementioned acceleration in the formation of chlorophyll *a*.
5. At very low intensities of light the chlorophyll formation follows a straight line and does not show any change in the rate of speed.

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References

1. Blaauw-Jansen, G., Komen, J. G. & Thomas, J. B.: On the relation between the formation of assimilatory pigments and the rate of photosynthesis in etiolated oat seedlings. — *Biochim. et Biophys. Acta* 5: 179. 1950.
2. Frank, Sylvia R.: The effectiveness of the spectrum in chlorophyll formation. — *Jour. Gen. Physiol.* 29: 157. 1946.
3. French, C. S. & Young, Violet K.: The fluorescence spectra of red algae and the transfer of energy from phycoerythrin to phycocyanin and chlorophyll. — *Jour. Gen. Physiol.* 35: 873. 1952.
4. - Towner, George H., Bellis, Donald R., Cook, Richard M., Fair, William R. & Holt, Walter W.: A curve analyzer and general purpose graphical computer. — *Rev. Sci. Instr.* 25: 765. 1954.

5. Koski, Violet M.: Gene action in relation to the development of chloroplast pigments in *Zea Mays* L. — Thesis, Univ. of Minnesota. U.S.A. 1949.
6. — Chlorophyll formation in seedlings of *Zea Mays* L. — Arch. Biochem. 29: 339. 1950.
7. — & Smith, James H. C.: The nature of the transformation of protochlorophyll to chlorophyll. — Carnegie Inst. of Wash. Year Book 48: 90. 1949.
8. — French, C. S. & Smith, James H. C.: The action spectrum for the transformation of protochlorophyll to chlorophylla in normal and albino corn seedlings. — Arch. Biochem. and Biophys. 31: 1. 1951.
9. Lubimenko, V. N. & Hubbenet, E. R.: The influence of temperature on the rate of accumulation of chlorophyll in etiolated seedlings. — New Phytologist 31: 26. 1932.
10. Noack, K. & Kiessling, W.: Zur Entstehung des Chlorophylls und seiner Beziehung zum Blutfarbstoffe. I. — Z. Physiol. Chem. 182: 13. 1929.
11. — Zur Entstehung des Chlorophylls und seiner Beziehung zum Blutfarbstoff. II. — Z. Physiol. Chem. 193: 97. 1930.
12. Rabinowitch, Eugene J.: Photosynthesis and related processes. I. — New York 1945.
13. Rudolph, H.: Ueber die Einwirkung des farbigen Lichtes auf die Entstehung der Chloroplastenfarbstoffe. — Planta 21: 104. 1933.
14. Schnarfngel, W.: Biologische Untersuchungen zur Chlorophyllbildung. — Planta 13: 716. 1931.
15. Seybold, A.: Zur Kenntnis des Protochlorophylls. III. — Planta 36: 371. 1948.
16. — & Egle, K.: Lichtfeld und Blattfarbstoffe. II. — Planta 28: 87. 1938.
17. Smith, James H. C.: Organic compounds of magnesium and phosphorus in relation to chlorophyll formation. — J. A. C. S. 69: 1492. 1947.
18. — Protochlorophyll, precursor to chlorophyll. — Arch. Biochem. 19: 449. 1948.
19. — Processes accompanying chlorophyll formation. — In »Photosynthesis in Plants». Ed. J. Franck and W. E. Loomis, Ames, Iowa. 1949.
20. — The relationship of plant pigments to photosynthesis. — Jour. Chem. Educ. 26: 631. 1949.
21. — The development of chlorophyll and oxygen-evolving power in etiolated barley leaves when illuminated. — Plant Physiol. 29: 143. 1954.
22. — & Benitez, Allen: The effect of temperature on the conversion of protochlorophyll to chlorophylla in etiolated barley leaves. — Plant Physiol. 29: 135. 1954.
23. — & Young, Violet K.: Chlorophyll formation and accumulation in plants. — In »Radiation Biology». Ed. A. Hollaender. In press.
24. Virgin, Hemming I.: The distortion of fluorescence spectra in leaves by light scattering and its reduction by infiltration. — Physiol. Plant. 7: 560. 1954.
25. — The conversion of protochlorophyll to chlorophylla in continuous and intermittent light. — Physiol. Plant. 8: 389. 1955.

The »Rhizosphere Effect» of Graminaceous Plants in Virgin Soils

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In an earlier investigation carried out in this laboratory (Gyllenberg, Hanioja, and Vartiovaara, 1954) it was observed that the vegetation obviously influences the numbers and kinds of bacteria in forest soils. This influence seemed to be most pronounced in forest soil types growing graminaceous plants in abundance. Later observations (Hanioja, Gyllenberg, and Vartiovaara, in preparation) have provided additional evidence in this respect. The literature reveals a good deal of information about the »rhizosphere effect» of higher plants on soil bacteria (cf. e.g. the review of Katznelson, Lochhead, and Timonin, 1948). Most of the investigations on this subject, however, have dealt with cultivated plants in tilled soils, and the knowledge of corresponding phenomena in virgin soils is quite meagre. The purpose of the present investigation was to obtain some information of the »autochthonous» bacteria in virgin soils, and specifically of the influence of graminaceous plants in this connection.

Methods

Four plants were selected for investigation, viz. *Phleum pratense*, *Poa pratensis*, *Anthoxanthum odoratum*, and *Deschampsia flexuosa*. »Rhizosphere samples» of these plants and samples of control soils were taken from five different sites. The sampling sites are described in Table 1. All the sites were located within a small area of approx. 4—5 acres.

The sampling was carried out during a few days around July 1st (1954). At this sampling the plants were in the blooming stage. Later, August 20th, additional

Table 1. *The source and designation of samples.*

Site	Description	pH	Samples collected
I	3hd year ley	6.5	July 1st, Control (CI) Phleum (PhI)
II	The narrow strip between arable land (site I) and forest (site III)	6.2	July 1st, Control (CII) Phleum (PhII) Poa (PII) Anthoxanthum (AII) Deschampsia (DII)
III	Edge of forest	5.8	July 1st, Control (CIII) Phleum (PhIII) Poa (PIII)
IV	Forest, growing pine	5.5	July 1st, Control (CIV) Phleum (PhIV) Anthoxanthum (AIV) Deschampsia (DIV) August 20th, Control (CIVa) Phleum (PhIVa) Anthoxanthum (AIVa) Deschampsia (DIVa)
V	Rock	4.8	July 1st, Control (CV) Deschampsia (DV)

samples were collected as indicated in Table 1. At this later sampling the plants were in the mature stage.

For the control samples pieces of soil (5×5×5 cm) were cut from each site. The plants and roots were extracted and the remaining soil was stirred carefully.

Several individuals of each plant were collected to obtain »rhizosphere samples». The adhering soil was removed from the roots by shaking. The shaking was continued until slight slapping against a white enamel dish gave no further visible soil from the roots. The roots were then washed in sterile water. The increase in volume resulting from suspending the soil in the water was noted, after which a suitable scheme was calculated for further dilution. In principle this method corresponds to those employed by other authors (e.g. Clark, 1939; Timonin, 1939).

For the platings, soil extract agar (West and Lochhead, 1940) was employed, and the plates were incubated for 14 days at 18° C before counting the colonies. 40—50 colonies were then taken from representative plates and transferred to semi-solid soil extract agar. By this procedure all the colonies from one dish, or all the colonies from a certain sector of a dish, were picked up. The cultures were examined microscopically, and on the basis of this examination the isolates were divided into actinomycetes, spore-forming bacteria, and non-sporogenous bacteria. Assuming that the percentual relations between these groups for the isolated cultures were valid for the initial sample also, the counts representing each group of bacteria could be obtained by calculation.

To express the effect of the plant roots on the distribution of bacteria in the soils the ratio — designed r/s -ratio — of the count (r) for each »rhizosphere sample» and that (s) for the corresponding control sample has been calculated.

228 cultures were selected from the isolated pure cultures for closer study. Of these, 177 represented non-sporogenous types and 51 spore-formers. These cultures were all isolated from samples representing sites II and IV (cf. Table 1).

The applicability of the method is a very important problem in »rhizosphere» investigations. The present study, however, is to be continued in order to obtain more information about the different factors influencing available methods. This subject is not, therefore, discussed here in more detail.

It may suffice to note that as regards the numbers of bacteria belonging to different systematic groups (actinomycetes, spore-formers, and non-sporogenous bacteria) the data are quite approximative. The calculations were based on the percentual distribution of some 40—50 cultures obtained from representative plates. This number may be considered inadequate (cf. Lochhead, 1952). The author tried, rather to obtain indicative data from a greater number of samples than to obtain more exact results from a few samples only. This could be realized only by limiting the number of cultures to be investigated.

Quantitative effect of the plant roots

The main results are presented in Table 2. As to the *total counts*, it can be seen that these were 11—31 times greater in the rhizosphere than in the corresponding control samples. It seems that *Phleum* and *Poa* exerted a somewhat greater effect than *Anthoxanthum* and *Deschampsia* under the same conditions. The effect of *Phleum* and *Poa* was, however, distinctly decreased when the conditions in the soil turned poorer (as indicated by lower pH values; Table 1), but *Anthoxanthum* and *Deschampsia* gave about the same r/s -ratios in all the soils investigated.

The influence of the plants on the *actinomycete* count was distinctly less significant than that on the total counts. The r/s -ratios ranged from 0 to 16. Here again, *Phleum* and *Poa* seemed to have exerted a greater influence, and the effect of *Anthoxanthum* and *Deschampsia* was quite insignificant.

As regards the counts of *spore-forming bacteria*, it was found that *Phleum* under the conditions of site I exerted a very pronounced promoting effect on these bacteria. *Phleum* and *Poa* had a considerable influence in site II also. In the samples representing *Phleum* and *Poa* in poorer conditions (sites III and IV) the effect of these plants was distinctly weaker. The effect of *Anthoxanthum* and *Deschampsia* remained rather weak in all the soils investigated.

In the r/s -values for *non-sporogenous bacteria* there seemed to be a smaller variation than in these ratios for the other groups of bacteria investigated.

Table 2. *The total counts, and the r/s-ratios for total counts, of actinomycetes, spore-formers, and non-sporogenous bacteria of the samples investigated.*

Sample	Total count		Actinomycetes r/s	Spore-formers r/s	Non-sporogenous bacteria r/s
	N/ $\text{cm}^3 \times 10^6$	r/s			
C I	14				
C II	18				
C III	16				
C IV	8				
C V	3				
Ph I	430	31	16	43	37
Ph II	350	20	8	21	29
Ph III	280	18	8	14	29
Ph IV	140	18	0	16	35
P II	440	24	10	24	38
P III	200	13	4	8	26
A II	200	11	2	5	24
A IV	80	11	0	3	32
D II	200	11	3	10	21
D IV	100	13	0	2	40
D V	50	19	0	2	55

The r/s-ratios ranged from 21 to 40 (with the exception of the *Deschampsia*-sample from site V; $r/s=55$). For all plants, and independently of the source of the samples, the r/s-ratios were of the same order of magnitude. Thus it can be concluded that all the plants investigated exerted a similar effect on the non-sporogenous bacteria, and that soil conditions do not influence this effect.

The second samplings provided data for a comparison of the »rhizosphere effect» of blooming and mature plants. The later series of sampling, however, concerned only 3 »rhizosphere samples», all from site IV: *Phleum*, *Anthoxanthum*, and *Deschampsia*. The results are presented in Tables 3 and 4.

Table 3. *The total counts and the corresponding r/s-ratios at different stages of plant development. (For designation of samples cf. Table 1).*

Sample	Total count (millions/ cm^3)	r/s-ratio
C IV	7.5	
C IVa	6.6	
Ph IV	135	18
Ph IVa	129	20
A IV	80	11
A IVa	102	15
D IV	95	13
D IVa	106.	16

Table 4. *The calculated counts of spore-formers and non-sporogenous bacteria, and the corresponding r/s-ratios at different stages of plant development. (For designation of samples cf. Table 1).*

Sample	Spore-formers		Non-sporogenous bacteria	
	Count $\times 10^6/\text{cm}^3$	r/s-ratio	Count $\times 10^6/\text{cm}^3$	r/s-ratio
C IV	3.6		2.2	
C IVa	3.7		2.7	
Ph IV	58	16	77	35
Ph IVa	19	5	110	41
A IV	11	3	69	32
A IVa	10	3	92	34
D IV	8	2	87	40
D IVa	6	2	100	37

As regards the total counts, neither the actual counts nor the r/s-ratios differ considerably from the figures obtained during the earlier stage of plant development. The most striking difference from the first sampling series was found in the spore-formers of the Phleum-sample. As shown in Table 4, the number of bacilli has dropped to about one third of the figure in the earlier sample, and the decrease in the r/s-ratio is to the same order of magnitude as found with *Anthoxanthum* and *Deschampsia* in all samples (cf. Table 2 also). The effect upon the non-sporogenous bacteria, however, has maintained the same level as in the earlier stage of development. This holds true for all the three plant species investigated. As regards the non-sporogenous bacteria, it appears as if the maturing of the plants would not result in a rapid decrease of the »rhizosphere effect». This finding agrees with the data presented by Starkey (1929) for biennial culture plants.

General characteristics of the isolated cultures

The determinative study of the *spore-forming* cultures revealed the fact that about 90 per cent of all the isolates were quite distinctly related to *Bacillus cereus* or *Bacillus cereus* var. *mycoides*. The other cultures were characterized by the formation of spherical and terminally located spores, thus obviously representing the *Bacillus sphaericus* group. However, cultures of the latter type were isolated from control soils only. Hence it can be concluded that the »rhizosphere effect» upon the spore-formers, observed for Phleum and Poa in some soils, was confined to *Bacillus cereus*.

Preliminary experiments on the nutritional requirements of the spore-formers revealed that the strains of *B. cereus* type are dependent on supplies

Table 5. *The nutritional requirements of some spore-forming strains.* (The basal medium contained 0.8 g KNO₃, 0.8 g KH₂PO₄, 0.1 g MgSO₄ · 7H₂O, 0.2 g NaCl, 0.02 g FeCl₃, and 2 g glucose in 1000 ml of distilled water; pH was adjusted to 6.8. As source of amino acids casein hydrolysate — »Vitaminfree Casamino acids», Difco — was added, and as source of vitamins a solution containing thiamin, biotin, p-aminobenzoic acid, folic acid, pyridoxin, and vitamin B₁₂. Pantothenic acid was tested separately. The growth figures were obtained turbidimetrically; a growth figure = 100 corresponds to the density-reading 0.5.)

	Medium				
	1.	2.	3.	4.	5.
Additions:					
Amino acids	—	+	—	+	+
Vitamin soln.	—	—	+	+	+
Pantoth. acid	—	—	+	+	—
Strain:					
<i>B. cereus</i> 215	5	70	6	74	
<i>B. cereus</i> 710	4	74	7	80	
<i>B. cereus</i> 1310	1	71	7	76	
<i>B. pantothenicus</i> 206	3	7	6	42	10

of amino acids for their growth. This finding agrees with the data given by Knight and Proom (1950). In addition to certain amino acids, pantothenic acid seemed to be an essential nutrient for the strains producing spherical spores, and it can be supposed, therefore, that these strains were related to *Bacillus pantothenicus*. (cf. eg. Smith, Gordon, and Clark, 1952). The growth figures obtained for some spore-forming strains in different media are presented in Table 5.

177 strains of *non-sporogenous* bacteria were subjected to closer study. The data obtained did not permit an accurate classification of the cultures investigated. It seemed, however, that Gram-negative types were more abundantly represented in the collection than were Gram-positive types. The major part of the Gram-negative strains may be organisms belonging to the genus *Pseudomonas*, and in addition to them obviously also organisms related to *achromobacteria* and *flavobacteria* were included in the collection. Most of the Gram-positive types again, showed properties which correspond to the typical characteristics of soil *corynebacteria*.

The number of strains of each type representing control samples and »rhizosphere samples», respectively, was too limited to provide a basis for recognizing a qualitative »rhizosphere effect» even where such an effect has occurred.

Discussion

The results of earlier authors (e.g. Starkey, 1929, Thom and Humfeld, 1932) show that the »rhizosphere effect» on the actinomycetes in general is less pronounced than on the eubacteria. This evidently holds good for the effect of the hay plants investigated, even in virgin soils. As to the spore-formers, it is reported that they do not multiply extensively in the rhizosphere (Krassilnikov, Kriss, and Litvinov, 1936, Starkey, 1938, Clark, 1939, Lochhead, 1940). The present investigation showed that this group is promoted under certain conditions, and by certain plants, but that the degree of the »rhizosphere effect» upon these bacteria in general is distinctly weaker than on the non-sporogenous bacteria.

The similar features of the »rhizosphere effect» on actinomycetes and on spore-formers is interesting. It seems that certain inhibiting environmental factors, e.g. the high acidity of some of the soils investigated, must be taken into consideration in discussing the »rhizosphere effect» on these groups. In acid environments the actinomycetes are inhibited, and sporulation of the bacilli is induced. Probably the »rhizosphere effect» thus partly arises from differences in prevailing environmental conditions, and not only from the excretion of essential nutrients by the roots. When environmental conditions change to unfavourable in the rhizosphere also, the actinomycetes are totally suppressed. The bacilli are maintained as spores, but are thus unable to take advantage of other beneficial factors.

Apart from the phenomena outlined above, the differences in the action of *Phleum* and *Poa*, on the one side, and *Anthoxanthum* and *Deschampsia*, on the other, must be noted. The present material, however, does not provide an explanation of the factors involved.

It is clearly shown that the most pronounced effect was upon the group of non-sporogenous bacteria. This result is in good agreement with data given by earlier investigators. The r/s-ratios, which represent virgin soils, may look low when compared with some corresponding figures for cultivated plants in tilled soils. The overall vegetation of the soils investigated must be considered in assessing this fact. This probably complicates the problem, since controls representing definite »non-rhizosphere» soil may be almost impossible to obtain.

It can be concluded that all the plants investigated exerted a fairly constant effect upon the non-sporogenous bacteria. The effect can be considered »constant» since its degree was independent of the plant species, the stage of plant growth, and the soil where the plants grew. Two plants, *Phleum* and *Poa*, showed a variable effect on actinomycetes and bacilli. The

»variability» of this effect is obvious from the fact that different plant species, the stage of plant development, and the conditions in the soil caused distinct variances in its degree.

Numerous earlier investigators have called attention to the difficulties occurring in connection with the classification of »autochthonous» non-sporogenous soil bacteria. As shown by Lochhead and his co-workers (West and Lochhead, 1940, Lochhead and Chase, 1943), the nutritional characteristics of these bacteria may provide a more accurate basis for the classification than the morphological and physiological properties ordinarily examined. A separate investigation of the nutritional features of the strains isolated during the course of the present study is in progress.

Summary

The »rhizosphere effect» of four graminaceous plants (*Phleum pratense*, *Poa pratensis*, *Anthoxanthum odoratum*, and *Deschampsia flexuosa*) has been investigated under the conditions prevailing in virgin soils. It was found that the number of bacteria was 11—31 times higher in the rhizosphere of these plants than in the corresponding »non-rhizosphere» control soils. The effect on non-sporogenous bacteria seemed to be constant since it was independent of the plant species, the stage of plant development, and the soil conditions. The promoting effect on spore-forming bacteria and actinomycetes again, varied with different plants, and showed dependence on the stage of plant development and the conditions prevailing in the soils.

The major part of sporogenous strains isolated were related to *Bacillus cereus*. This type was predominating in the »rhizosphere samples», but in the control samples also types related to *Bacillus pantothenicus* were found. Among the non-sporogenous isolates Gram-negative types were somewhat more abundant than Gram-positive types. The Gram-negative strains obviously belonged to the genera *Pseudomonas*, *Achromobacter*, and *Flavobacterium*, and the Gram-positive to the genus *Corynebacterium*, respectively.

References

- Clark, F. E.: Effects of soil amendments upon the bacterial population associated with roots of wheat. — Trans. Kan. Acad. Sci., 42: 91. 1939.
- Gyllenberg, H., Hanioja, P. & Vartiovaara, U.: Observations on the composition of the microbial population in some virgin soils (English Summary). — Acta Forest. Fenn., 62: 1. 1954.
- Hanioja, P., Gyllenberg, H., & Vartiovaara, U.: In preparation.

- Katznelson, H., Lochhead, A. G., & Timonin, M. I.: Soil microorganisms and the rhizosphere. — *Botan. Rev.*, 14: 543. 1948.
- Knight, B. C. & Proom, H.: A comparative survey of the nutrition and physiology of mesophilic species in the genus *Bacillus*. — *Jour. Gen. Microbiol.*, 4: 508. 1950.
- Krassilnikov, N. A., Kriss, A. E., & Litvinov, M. A.: The microbiological characteristics of the rhizosphere of cultural plants (English Summary). — *Mikrobiologija*, 5: 87. 1936.
- Lochhead, A. G.: Qualitative studies of soil microorganisms. III. Influence of plant growth on the characteristics of the bacterial flora. *Can. Jour. Res., C*, 18: 42. 1940.
- The nutritional classification of soil bacteria. — *Proc. Soc. Appl. Bacteriology*, 15: 15. 1952.
- & Chase, F. E.: Qualitative studies of soil microorganisms. V. Nutritional requirements of the predominant bacterial flora. — *Soil Sci.*, 55: 185. 1943.
- Smith, N. R., Gordon, R. E., & Clark, F. E.: Aerobic spore-forming bacteria. — U. S. Dept. of Agriculture, Washington, pp. 142. 1952.
- Starkey, R. L.: Some influences of the development of higher plants upon the microorganisms in the soil. II. Influence of the stage of plant growth upon the abundance of organisms. — *Soil Sci.*, 27: 355. 1929.
- Some influences of the development of higher plants upon the microorganisms in the soil. VI. Microscopic examination of the rhizosphere. — *Soil Sci.*, 45: 207. 1938.
- Thom, C. & Humfeld, H.: Notes on the associations of microorganisms and roots. — *Soil Sci.*: 34: 29. 1932.
- Timonin, M. I.: The interaction of higher plants and soil microorganisms. Thesis, Rutgers University 1939 (ref. by Katznelson et al., 1948).
- West, P. M. & Lochhead, A. G.: Qualitative studies of soil microorganisms. IV. The rhizosphere in relation to the nutritive requirements of soil bacteria. — *Can. Jour. Res., C*, 18: 129. 1940.

Studies on Nitrogen-fixing Blue-green Algae

II. The Sodium Requirement of *Anabaena cylindrica*

By

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Introduction

The element sodium has never been shown to be indispensable for the growth of green plants in accordance with a definite set of criteria of essentiality (4). Although the quantity of potassium required by some plants has been reported to be reduced in the presence of sodium, this element cannot replace potassium completely. The omission of sodium from the nutrient medium in the presence of adequate potassium has not been conclusively shown to have any detrimental effect on plant growth (6).

However, there have been various indications that sodium might play a more important role in the nutrition of one group of lower plants, the blue-green algae (Myxophyceae). This evidence has been of two types, the first suggesting that sodium and potassium are readily interchangeable in the nutrition of these algae, and the second pointing toward a specific sodium requirement regardless of potassium status. In 1898 Benecke (7) described an *Oscillatoria*, a member of the Myxophyceae, which grew in a medium in which all the potassium salts had been replaced by sodium compounds. Emerson and Lewis (8) studied a *Chroococcus* species which grew, in the presence of sodium, with only 1 part per million potassium added to the culture medium. Allen (1) found that 23 cultures of various Myxophyceae behaved in the manner described by Benecke, growing in sodium salt media without added potassium. The Emerson and Lewis *Chroococcus* grew little, if at all, when sodium was omitted from the medium (8). Several of Allen's

cultures behaved in a similar fashion (1). Gerloff, Fitzgerald, and Skoog (9) reported a beneficial effect of sodium carbonate or sodium silicate on the growth of *Microcystis aeruginosa*, but it was not clear in these experiments whether the effect of these salts was due to their sodium content or to the increase in pH produced by them. Kratz and Myers (11) have recently reported a marked effect of sodium on the rate of growth of *Anacystis nidulans*.

Most of the observations cited above were qualitative or semi-quantitative, and based on experiments carried out with unpurified salts, undoubtedly contaminated with other alkali metals. Nevertheless, these results were sufficiently suggestive to make desirable a critical examination of sodium as a nutrient for blue-green algae.

Methods

The alga used in these experiments was a pure culture of Fogg's strain of *Anabaena cylindrica* Lemm. The medium for its culture and the experimental arrangements have been described in detail elsewhere (2, 5). The constituents of the nutrient solution were MgSO_4 , 0.001 *M*; KCl, 0.004 *M*, K_2HPO_4 , 0.002 *M*; and NaCl as specified. The high potassium concentration was used to ensure that the effects observed did not result from a sparing effect of sodium on potassium. Molecular nitrogen served as nitrogen source in most of the experiments; when it was desired to grow the cultures with combined nitrogen potassium nitrate (0.020 *M*) was added to the medium. A micronutrient supplement based on the A4 and B6 solutions (2, 3, 5) was added in all experiments.

To guard against the possibility that the effects ascribed to sodium might be due to contamination of the sodium chloride with an unknown micronutrient element, several elements not so far shown to be required by algae or other plants were also added to the nutrient solution (cf. 2).

In order to observe clear-cut effects of sodium on the algal cultures it was necessary to take precautions to exclude sodium contamination from the glassware and reagents used. Glass-distilled water was used for all media. All glassware was washed with 3 *N* HCl, followed by thorough rinsing with glass-distilled water, to remove traces of sodium left by the detergent used for dishwashing. C.P. grade MgSO_4 , KCl, NaCl, K_2HPO_4 and Na_2HPO_4 were purified by three successive recrystallizations from hot water. CaSO_4 was prepared by addition of H_2SO_4 to a solution of calcium chloride, followed by thorough washing of the CaSO_4 precipitate.

Growth was measured by removing aliquots of the cultures, evaporating to dryness at 80–90° C, and applying a correction for the weight of the

salts in the medium (2). For measurement of chlorophyll the cells were extracted with hot methanol and the optical density of the methanolic extract determined at 665 m μ with a Beckman spectrophotometer. From this figure and the known extinction coefficient of chlorophyll at this wave length (10) the amount of chlorophyll was calculated. Phycocyanin was estimated in an aqueous extract obtained by repeated freezing and thawing of the cells. The optical density of this solution at 620 m μ was measured and the phycocyanin content calculated from the extinction coefficient of phycocyanin (13).

Results and Discussion

In order to be considered as essential an element must meet the following criteria: normal plant growth must be impossible in its absence; the deficiency symptoms caused by absence of the element must be removed on its addition to the medium; the requirement must be specific, it must not be possible to substitute any other nutrient for the element in question; and lastly its function or at least its direct effect on the metabolism of the plant must be identified (4).

By taking precautions to exclude sodium contamination it has been possible to show that sodium meets these criteria for *Anabaena cylindrica*.

Normal development of the alga did not occur in the absence of sodium; the appearance of cultures with and without added sodium is shown in Figure 1. The scanty growth obtained in the absence of sodium was restored to the normal level by addition of this element, as indicated in Figure 2. The 'first transfer' illustrates the results obtained with and without added sodium

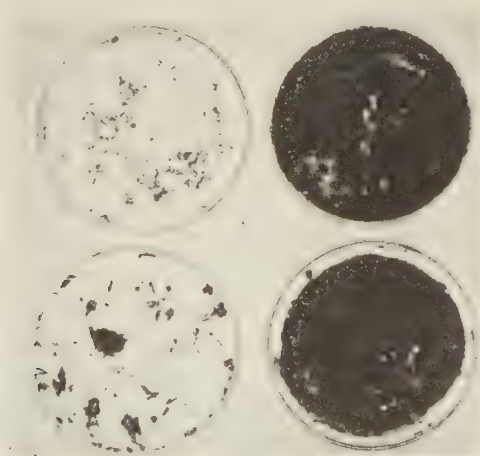


Figure 1. *Effect of sodium on the growth of Anabaena cylindrica*. Pale cultures — no sodium added; dark cultures received 92 mg. Na per liter. N₂ was the source of nitrogen.

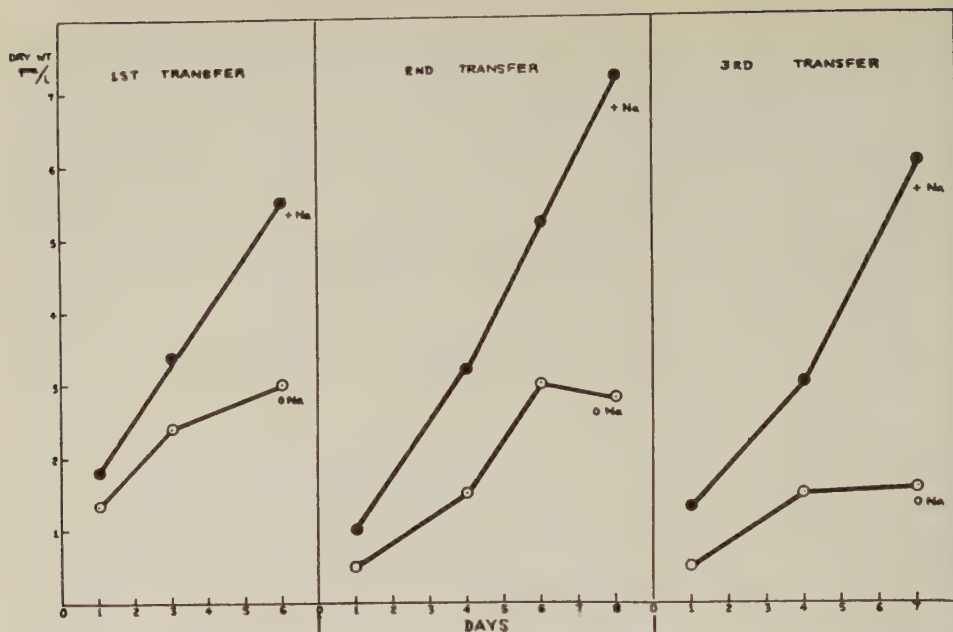


Figure 2. Recovery of sodium-deficient cultures of *Anabaena cylindrica* in three successive transfers to a nutrient solution containing 92 mg. Na per liter. N_2 was the source of nitrogen.

when inoculum from a plus-sodium parent culture was used. In the succeeding experiment, inoculum from the sodium-deficient culture was used in media with and without added sodium, giving the results shown as 'second transfer' (Figure 2). The alga recovered and grew well when sodium was added, but grew very little in the absence of sodium. The difference between cultures with and without sodium became even greater when the procedure was repeated, as shown for the 'third transfer' (Figure 2).

The dependence of *Anabaena* growth on the quantity of sodium added is illustrated in Fig. 3. Five parts per million was found to be adequate for optimal development of the alga. There is no indication that larger amounts of sodium are harmful.

The requirement for sodium was found to be specific. In the sodium concentration series (Figure 3), potassium nitrate was used as a source of nitrogen, supplying 20 milliequivalents of potassium to the minus-sodium culture. It is evident, therefore, that sodium was not replaceable by potassium. Lithium, rubidium or cesium were similarly ineffective as a substitute for sodium (Figure 4). Further experiments, illustrated in Figure 5, have shown that just as sodium was required by *Anabaena* in the presence of adequate potassium, so was potassium indispensable in the presence of adequate

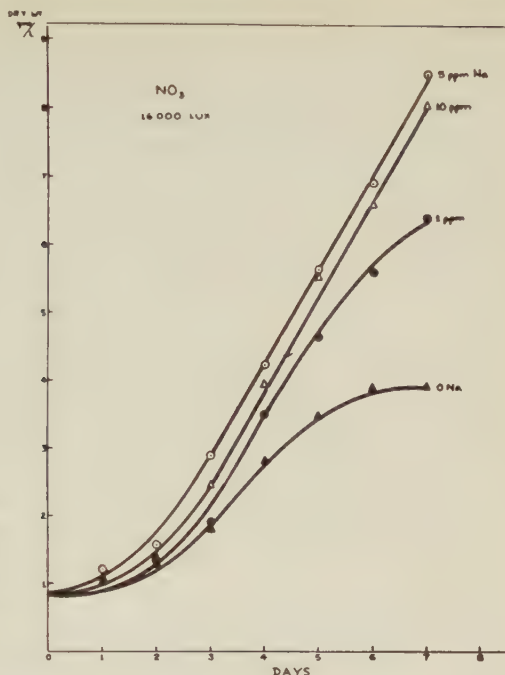


Figure 3. Effect of sodium concentration on the growth of *Anabaena cylindrica* in the presence of potassium (860 mgs. K as KNO_3 per liter of nutrient solution). Similar results were obtained with N_2 as nitrogen source.

sodium. The slightly depressed growth in the cultures containing 1 part per million of lithium, rubidium, or cesium might be due to toxicity of these metals but is more likely the result of experimental error, since raising their

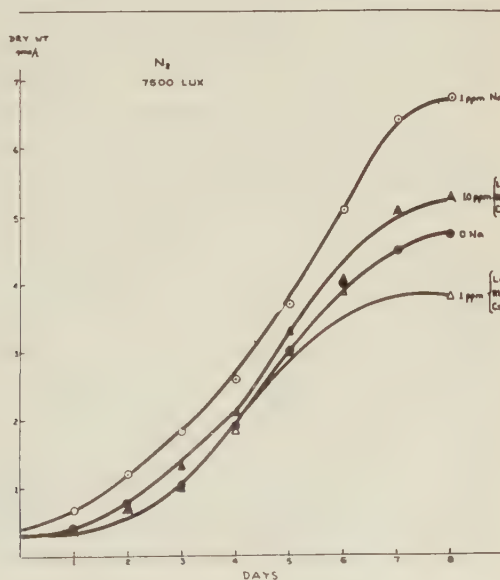


Figure 4. Failure of lithium, rubidium and cesium to substitute for sodium in the growth of *Anabaena cylindrica*.

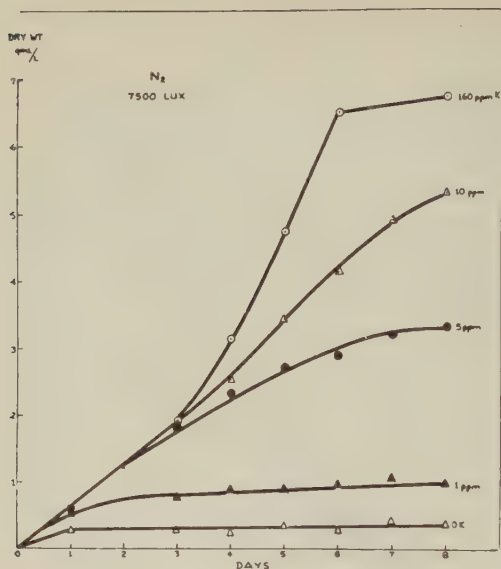


Figure 5. Effect of potassium concentration on the growth of *Anabaena cylindrica* in the presence of sodium (92 mg Na per liter).

concentration to 10 parts per million did not result in a further decrease of growth.

Only limited information is so far available on the function of sodium in the metabolism of *Anabaena*. One noticeable effect of sodium deficiency was that the cultures were yellowish-green, in contrast to the deep blue-green color characteristic of *Anabaena cylindrica* in a healthy condition. As shown in Table 1, this change in color was due to the lower phycocyanin content of the sodium deficient cultures; chlorophyll was not significantly affected. Although the absolute amount of phycocyanin in the cells varied greatly with the age of the culture and the light intensity used for growth, for each set of conditions the phycocyanin content of cells grown without added sodium was markedly lower.

One other effect of sodium on blue-green algae, which may or may not be related to its role in the development of these organisms, deserves mention. In order to maintain a steady high rate of photosynthesis in resting cells it is necessary to suspend the cells in a buffer containing sodium salts. This effect of sodium, first observed on *Synechococcus cedrorum* (1), has been noted for *Anabaena* as well. Sodium is needed in the suspension medium even when the cells have been grown in a culture medium with adequate sodium. This behavior of blue-green algae is in contrast to that of *Chlorella*. With this alga photosynthesis declines in cells suspended in buffers composed of sodium salts but remains constant for several hours when potassium salts are used in the suspension medium (12).

Table 1. *Effect of sodium deficiency on pigmentation of Anabaena cylindrica*. The concentration of sodium in the +Na cultures was 92 mg. per liter (p.p.m.). Pigment content expressed as per cent of dry weight of algal cells.

Expt. No.	N Source	Light Intensity	Age of Culture	Per cent phycocyanin		Per cent chlorophyll	
				— Na	+ Na	— Na	+ Na
60	NO ₃	7,500 lux	10 d.	0.02	0.45	0.12	0.17
61	N ₂	7,500 "	8 d.	0.63	1.60	0.15	0.25
68	NO ₃	16,000 "	9 d.	0.06	0.12	0.20	0.23

Although essentiality of sodium has been proven only for one member of the Myxophyceae, *Anabaena cylindrica*, the various qualitative observations of beneficial effects of sodium on other blue-green algae make it likely that a sodium requirement is a general characteristic of these organisms.

Summary

Sodium has been shown to be essential for growth of the blue-green alga *Anabaena cylindrica*. A sodium concentration of 5 ppm or higher is required for optimal growth of this alga. The sodium requirement is specific; potassium, lithium, rubidium, and caesium can not substitute for sodium. Similarly, sodium cannot replace potassium for growth of *Anabaena*.

Sodium-deficient cultures were shown to contain less phycocyanin than those with adequate sodium. Their chlorophyll content was not affected.

References

1. Allen, M. B.: The cultivation of Myxophyceae. — Arch. f. Mikrobiol. 17: 34. 1952.
2. Allen, M. B. & Arnon, D. I.: Studies on nitrogen-fixing blue-green algae. I. Growth and nitrogen fixation by *Anabaena cylindrica*. — Plant Physiol. in press.
3. Arnon, D. I.: Microelements in culture-solution experiments with higher plants. — Amer. J. Bot. 25: 322. 1938.
4. — Growth and function as criteria in determining the essential nature of inorganic nutrients. — In Mineral Nutrition of Plants. pp. 313—341 E. Truog, Ed. Univ. of Wisconsin Press, Madison Wis. 1952.
5. Arnon, D. I., Ichioka, P. S., Wessel, G., Fujiwara, A. & Woolley, J. T.: Molybdenum in relation to nitrogen metabolism. I. Assimilation of nitrate nitrogen by *Scenedesmus*. — Physiol. Plant. 8: 538. 1955.
6. Bear, F. E.: Ed. Sodium symposium. — Soil Science 76: 1. 1953.
7. Benecke, W.: Über Culturbedingungen einiger Algen. — Bot. Zeit. 56: 83. 1898.
8. Emerson, R. & Lewis, C. M.: The photosynthetic efficiency of phycocyanin in *Chroococcus* and the problem of carotenoid participation in photosynthesis. — J. Gen. Physiol. 25: 579. 1942.

9. Gerloff, G. C., Fitzgerald, G. P. & Skoog, F.: The mineral nutrition of *Microcystis aeruginosa*. — *Am. J. Bot.* 39: 26. 1952.
10. Harris, D. G. & Zscheile, F. P.: Effects of solvent upon absorption spectra of chlorophylls a and b. — *Bot. Gaz.* 104: 515. 1943.
11. Kratz, W. & Myers, J.: Nutrition and growth of several blue-green algae. — *Am. J.* 42: 282. 1955.
12. Pratt, R.: Studies on *Chlorella vulgaris* VIII. Influence on photosynthesis of prolonged exposure to sodium bicarbonate and potassium bicarbonate. — *Am. J. Bot.* 30: 626. 1943.
13. Svedberg, T. & Katsurai, T.: The molecular weights of phycocyan and of phycoerythrin from *Porphyra tenera* and of phycocyan from *Aphanizomenon flos-aquae*. — *J. Am. Chem. Soc.* 51: 3573. 1929.

The Influence of Some Inorganic Salts on the Sporulation of a Strain of *Bacillus stearothermophilus*

By

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During some determinative studies on thermophilic bacteria of the genus *Bacillus* Cohn, it was observed that the sporulation of one strain, called 234A, was greatly increased by the addition of 0.1 per cent of NaNO_3 to the medium (nutrient agar), on which the organisms were grown. This fact was interesting because very few spores had been observed in the stained smears prepared from cultures grown on nutrient agar without nitrate added. The strain 234A was isolated by the author from spoiled canned peas (received from AB Findus, Bjuv), and was identified as being closely related to *Bacillus stearothermophilus* Donk. As mentioned above, it was observed that sporulation was greatly increased by the addition of NaNO_3 to the nutrient agar used in the determinative studies. Because of these observations a series of experiments were made in order to study more closely the effect of nitrate on the sporulation of 234A.

There is a copious literature on endospore formation in bacteria and numerous investigations have been carried out. A great deal of these have been concerned with the influence of various substances on the sporulation of bacteria. Many workers have tried to find relations between various concentrations of nutrients in the medium and the percentage of spores formed. There are also studies on the effect of different concentrations of various salts in the medium on the sporulation.

In all papers read by the author while searching for investigations of the same type as his own, only cations such as Na^+ and Li^+ have been studied and nothing has been written about the influence of anions on the sporula-

tion of bacteria. Moreover, all these investigations have been carried out with *mesophilic* organisms and the *thermophilic* bacteria seem to have been completely neglected.

In his review Gaughran (1947) says that thermophilic bacteria often sporulate very little and sometimes the sporulation may be so bad that the cultures die out if stored at elevated temperatures. This is in harmony with the author's own observations on 234A. Knaysi (1948) has a review on most of the work done on endospore formation in bacteria, but nowhere in this review anything is mentioned about thermophilic bacteria and their sporulation. About the influence of anions nothing is mentioned.

Fabian and Bryan (1933) investigated the influence of the cations, K^+ , Na^+ , Li^+ and NH_4^+ on the sporulation of four aerobic mesophilic bacteria and they concluded that these ions had a stimulating effect on the sporulation. The only worker who has made experiments of the same type as the present author's, is Schmidt (1950). He writes that there are almost no investigations at all of the sporulation problems of the thermophilic bacteria. Schmidt has worked with »flat sour» organisms of the same type as 234A. He has not studied the influence of anions but the effect of different concentrations of peptone, NaCl and $CaCl_2$ in a solid agar medium. He observed that different concentrations of peptone in the medium produced different percentages of spores in the cultures. He also found that NaCl had no positive effect on the sporulation, in some cases the sporulation rather seemed to be depressed by this salt. Schmidt states that one is restricted to say that the results obtained are representative only for the organism used in the studies and that these results cannot be generalized because too little is known about the factors influencing spore formation in general. No later articles than Schmidt's on this subject have been found.

Material and methods

A pure culture, called 234A, was used in the experiments. The methods used for the identification of the bacteria were taken from Manual of Methods for Pure Culture Study of Bacteria (1949), leaflets II, IV and V, and Smith, Gordon, and Clark (1952). The general properties of strain 234A are given below. In the identification of the species these properties were compared with those listed in Bergey's Manual (Breed et al. 1948, 6th ed.) and in Smith, Gordon and Clark (1952).

Summarized description of the strain 234A

Spores: Ellipsoidal, 0.8—0.9 by 1.5—1.8 microns, terminal to subterminal. Sporangia: Occurring singly, not in chains, swollen, drumstick-shaped.

Rods: 2—5 by 0.6—0.8 microns, sometimes occurring in chains, sometimes not. Nonmotile, Gram-positive.

Agar colonies: Small, circular, convex, butyrous, edge entire, often pinpoint-type.

Growth in broth: slightly turbid, growth not very abundant.

Nitrite produced from nitrate.

Gelatin not hydrolyzed.

Starch is hydrolyzed.

Acid produced without gas from glucose.

Litmus milk unchanged.

Indole not produced.

Casein not hydrolyzed.

Slight growth at 40° C, no growth at 37° C. The temperature optimum is about 55° C. Maximum temperature about 65—70° C.

234A resembles in most respects *Bacillus stearothermophilus* Donk although some differences exist as regards the motility and gelatin hydrolysis. This species is the type species of the «flat sour» organisms, which are often found in spoiled canned peas.

The culture tubes were always inoculated from a 24 hours old culture, grown at 55° C. The bacteria were grown on agar slants and two series of tubes of the same kind were prepared: one was incubated at 45° C and the other at 55° C. Each series comprised five different concentrations of the salt in question, added to the nutrient agar. Some tests were made at 65° C but the growth was so poor that the results cannot be considered here. For the nutrient agar the following ingredients were used: Bacto Beef Extract 3 g; Bacto Peptone 5 g; Bacto Yeast Extract 5 g; Bacto Agar 15 g; Distilled water 1000 ml. Sterilization was carried out at 120° C for 20 minutes. Pyrex glassware was used throughout. The following salts were used in the experiments: KNO_3 , KNO_2 , NaNO_3 , NaNO_2 , KCl , NaCl , and a mixture of 2 parts of NaNO_3 + 3 parts of NH_4Cl . One series with $\text{NH}_2\text{OH}\cdot\text{HCl}$ was made but this salt had only a toxic effect and the result will not be included here.

Stainings and microscopic studies were made after 24 and 48 hours of incubation. The spore stainings were made with carbol fuchsin and methylene blue

From every tube two smears were prepared and stained and as there were two tubes for each concentration and temperature, four smears were prepared from each salt concentration and for each temperature. After the staining of the prepared smears, about 800 (vegetative cells + spores) were counted for each salt concentration and for each temperature. Counts were made after 24 and 48 hours of incubation. This method for counting is rather approximate but it has been considered accurate enough for the purpose of these investigations. The Tables show that it has been quite enough to demonstrate the existing differences.

The number of spores have been expressed as per cent of the total number of (vegetative cells + spores) for each count. The values thus obtained have been rounded off to whole numbers with the exception of some very low values where the values have been set to 0.5 per cent. The adjustments of the percentage num-

bers are accounted for partly by the methods used for counting the spores and cells, partly by the introductory character of the experiments.

The results of the counts are given in the Tables 1—5. In these Tables the different salt concentrations are given in per cent and the observations after 24 and 48 hours at 45° C and 55° C are given as per cent of spores of the total number of (cells+spores).

Results and Discussions

The Tables clearly show that nitrate and nitrite certainly do exert a stimulating effect on the sporulation of 234A, but some irregularities can be noted regarding the action of different salts (mainly differences between KNO_3 and KNO_2 , NaNO_3 , NaNO_2). Also different concentrations have different effects.

As has been mentioned before, $\text{NH}_2\text{OH}\cdot\text{HCl}$ has been tested but as the only result was a poisoning of the culture, the result will not be considered here. The reason why nitrite and $\text{NH}_2\text{OH}\cdot\text{HCl}$ were tested was that it is generally accepted that at the nitrate reduction in higher plants first nitrite is formed and then possibly NH_2OH is a later intermediate product. The author therefore wanted to investigate if these substances would have any positive effect on the sporulation as it was clearly demonstrated that nitrate had a stimulating influence. Strain 234 produces nitrite from nitrate. Anyhow, very little is known about the nitrate assimilation in bacteria and nothing can be said about the intermediate products after the nitrite stage.

From the tables 1 and 2 it is clear that differences exist between effective concentrations of KNO_3 and NaNO_3 respectively and that higher concentrations of KNO_3 are most favourable at 45° C and that the best sporulation is brought about by lower concentrations of NaNO_3 at 55° C. The tables show that nitrate in the form of KNO_3 or NaNO_3 definitely has a positive effect on the spore formation of 234A. It can be noticed that at higher con-

Table 1. *The effect of KNO_3 .*

% KNO_3	Per cent of spores			
	45°		55°	
	24 hrs	48 hrs	24 hrs	48 hrs
0.00	0	0	0	0
0.01	1	8	0.5	1
0.05	6	18	7	5
0.1	25	28	15	9
0.2	21	22	9	6
0.5	12	20	6	4

Table 2. *The effect of NaNO₃.*

% NaNO ₃	Per cent of spores			
	45°		55°	
	24 hrs	48 hrs	24 hrs	48 hrs
0.00	0	0	0	0
0.01	0	0,5	16	12
0.05	1	3	18	20
0.1	0	0,5	19	15
0.2	3	1	6	4
0.5	0	0	2	0

centrations of NaNO₃ the growth was not very good and the cells did not stain so well.

The tables 3 and 4 show the effect of nitrite on the sporulation. The positive effect seems to be greatest at the lowest concentrations and at 55° C and NaNO₂ seems to have a stronger effect than KNO₂. At higher concentrations of nitrite the growth was very poor.

Because of the fact that the best sporulation is obtained at the lowest concentrations of nitrite and at much higher concentrations of nitrate it is possible that sporulation is stimulated just when nitrate is reduced to nitrite and that nitrite (or products later formed from nitrite) may be the factor stimulating the sporulation. According to this theory the results obtained with NaNO₂ seem to be in harmony with those obtained with NaNO₃. The relations between the results with KNO₃ and KNO₂ are not so clear, the places for the highest values are not the same in the two tables. This fact may be due to some unknown experimental errors but it might as well be due to some special effect of KNO₃. The relations between sporulation and the presence of these salts certainly are much more complex than can be shown by these simple experiments.

Some control tests were made with KCl and NaCl, the concentrations used

Table 3. *The effect of KNO₂.*

% KNO ₂	Per cent of spores			
	45°		55°	
	24 hrs	48 hrs	24 hrs	48 hrs
0.00	0	0	0	0
0.01	0	0	18	15
0.05	1	0.5	10	6
0.1	0	0	4	5
0.2	0	0	0	0
0.5	0	0	0	0

Table 4. *The effect of NaNO₂.*

% NaNO ₂	Per cent of spores			
	45°		55°	
	24 hrs	48 hrs	24 hrs	48 hrs
0.00	0	0	0	0
0.01	1	18	23	19
0.05	1	12	20	12
0.1	0	1	10	9
0.2	0	0	4	1
0.5	0	0	0	0

were the same as in the other experiments and these tests were made exactly as described above. These tests were made in order to study if K⁺-ions or Na⁺-ions would have any influence on the sporulation. These tests were considered necessary to ascertain that it was the anions (nitrate and nitrite) that stimulated the sporulation in the previous experiments. The result was that no influence could be detected; the sporulation was very slight or in most cases absent. This is in harmony with the results of Schmidt; he too found no effect of NaCl. The results discussed above show, that there is every probability that the sporulation of 234A is positively influenced by the addition of nitrate or nitrite to the medium, and that the effect of nitrate is very possibly due to its reduction to nitrite. The action of nitrite may be due to some substance later formed from it, but the investigations are not carried further than to the »nitrite stage».

Table 5 shows the results obtained when different concentrations of (NaNO₃+NH₄Cl) were added to the medium. The mixture consisted of 2 parts of NaNO₃ and 3 parts of NH₄Cl. The reason for these tests was that it has been observed in higher plants that the nitrate uptake some times is reduced by the addition of ammonium ions to the medium. However, this phenomenon is, as far as the author knows, very little or perhaps not at all

Table 5. *The effect of NaNO₃+NH₄Cl.*

% NaNO ₃	% NH ₄ Cl	Per cent of spores			
		45°		55°	
		24 hrs	48 hrs	24 hrs	48 hrs
0.000	0.000	0	0	0	0
0.010	0.015	2	6	10	5
0.050	0.075	0	1	12	14
0.100	0.150	0.5	1	9	11
0.200	0.300	0	0	8	2
0.500	0.750	0	0	0	0.5

known in bacteria. The author's intentions have been to investigate if the addition of ammonium ions (in the form of NH_4Cl) would repress or reduce the stimulating effect of nitrate ions. This would perhaps be the case if the nitrate uptake was reduced or inhibited by NH_4Cl as has been demonstrated with some higher plants. A look at the tables 2 and 5 will show that there is almost no difference between the effect of nitrate in the presence or absence of NH_4Cl . The somewhat lower values found in table 5 may be ascribed to variations in the cultures, depending partly on the organisms themselves, partly on minor variations that may have occurred in the experimental conditions. The author agrees with Schmidt, who says, that in a population of bacteria there seems to be a smaller or greater percentage of individuals which are more liable to produce spores as a response on changes in the environment, e.g. when salts of different kinds are added to the medium in which the bacteria are grown. In the tables 2 and 5 the highest values are found in the same places, viz. at 55°C and 0.01—0.1 per cent of salt. The result of this last experiment is, that the ammonium chloride has had no influence on the stimulating effect of nitrate on the sporulation of 234A.

Summary

One strain of thermophilic bacteria has been isolated from spoiled canned peas and identified as being closely related to *Bacillus stearothermophilus* Donk.

This strain, called 234A, has been used for some investigations of the influence of nitrate on the sporulation of this kind of bacteria. At the same time the effect of nitrite has been studied and finally some tests have been carried out in order to ascertain if ammonium ions (in the form of NH_4Cl) in some way would change the effect of nitrate.

The results clearly show that nitrate, possibly after reduction to nitrite, has a stimulating effect on the sporulation of 234A, although the nature of this effect is still quite unknown.

Control tests made with KCl and NaCl have shown that no stimulation is obtained with these salts, viz. with potassium or sodium ions. No depression of the positive effect of nitrate could be observed in the presence of NH_4Cl , viz. ammonium ions.

The experiments and results must be looked upon as being only preliminary and qualitative although some quantitative relations seem to exist between the concentration of nitrate (or nitrite) in the medium and the percentage of spores formed. The greatest percentage of spores seems to be obtained at about 0.1 per cent of nitrate and at 0.01—0.05 per cent of nitrite.

Further investigations are needed on the factors influencing the sporulation of bacteria, both thermophilic and mesophilic.

I am indebted to Professor Hans Burström who placed laboratory accomodation and equipment at my disposal. I also want to thank Dr Gösta Fåhraeus for valuable discussions, advice and criticism.

References

- Breed, R. S. et al.: *Bergey's Manual of Determinative Bacteriology*. — 6th ed., London. 1948.
- Fabian, F. W. & Bryan C. S.: The Influence of Cations on Aerobic Sporogenesis in a Liquid Medium. — *J. Bact.* 26: 543. 1933.
- Gaughran, E. R. L.: The Thermophilic Microorganisms. — *Bact. Revs.* 11: 189. 1947.
- Knaysi, G.: The Endospore of Bacteria. — *Bact. Revs.* 12: 19. 1948.
- Schmidt, C. F.: Spore Formation by Thermophilic Flat Sour Organisms. I. The Effect of Nutritient Concentration and the Presence of Salts. — *J. Bact.* 60: 205. 1950.
- Smith, N. R., Gordon, R. E. & Clark, F. E.: *Aerobic Sporeforming Bacteria*. — U.S. Dept. Agr.; Agr. Monograph 16. 1952.
- Society of American Bacteriologists: *Manual of Methods for Pure Culture Study of Bacteria*. — Leaflets II, IV, and V. 1949.

The Effect of Gibberellic Acid on Shoot Growth of Pea Seedlings

By

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Introduction

Gibberellic acid is a metabolic product of the fungus *Gibberella fujikuroi*; it possesses marked plant growth promoting properties (Curtis and Cross, 1954; Brian *et al.*, 1954; Cross, 1954; Mulholland and Ward, 1954; Borrow *et al.*, 1955). It is distinct from but chemically related to gibberellin A, a substance with similar physiological properties isolated from cultures of the same fungus by Yabuta and Hayashi (1939). The gibberellin X of Stodola *et al.* (1955) and gibberellic acid are identical. References to numerous Japanese papers on gibberellin A are given in the papers on gibberellic acid quoted above. Gibberellic acid differs physiologically from most auxins hitherto described. Whereas it is usually much less active than they are in assays based on a response of isolated plant parts, it stimulates the growth of intact plants much more vigorously than they do, substantially increasing height, fresh weight and dry weight (Brian *et al.*, 1954). In this communication we give a more quantitative analysis of the relation between the dose applied and the growth response of pea seedlings as measured by increase in shoot height. A differential response in a range of varieties of pea is also described.

Materials and Methods

Experimental plants

Pea seedlings were grown in John Innes Compost (No. 2) in 400 ml. glass beakers, four seedlings in each beaker. Watering was carefully regulated and no trouble with waterlogging was experienced. The plants were grown in cool glasshouse with supplementary lighting from fluorescent strip lamps in winter. All experiments were replicated and arranged in randomised blocks on the glasshouse bench. The varieties of pea used are listed in Table 2.

Application of gibberellic acid

In the first experiment described, gibberellic acid was applied in ethyl alcohol solution. Each plant received 0.002 ml. of solution, delivered from a micropipette ('Agla' brand micrometer syringe); this was applied to one of the leaflets of the first true leaf. Preliminary experiments had shown that no damage was caused by such quantities of alcohol and that growth rate was unaffected by alcohol alone; it had been found too that gibberellic acid was translocated from the first leaf, as judged by an increased rate of extension of upper internodes first detectable 24 hours after application. This method enables accurately measured doses to be applied.

In the second experiment aqueous solutions of gibberellic acid and indolylacetic acid were applied as a spray until just before run-off.

Growth measurements

Measurements of shoot height were made to the nearest millimetre at the beginning of the experiment and at suitable times thereafter. Either the total height from the node bearing the first true leaf, or each separate internode above that node (i.e. internode 4 and later ones) were measured. Under the experimental conditions chosen no extension growth of internodes below internode 4 took place after treatment of the plants. For statistical treatment the growth increments of the four seedlings in each beaker were added, each beaker being treated as a single observation, but the results are presented as mean growth increments for a single plant.

Results

Dose-response relations

In this experiment three varieties of pea were used: — Meteor, Duke of Albany and Improved Pilot. Plants were treated by the ethyl alcohol microdrop method with gibberellic acid in doses increasing by twofold steps from 0.01 μ g. to 10.24 μ g. per plant. Seven replicate beakers, each containing 4 seedlings were used for each treatment. Internode 4 and all internodes above were measured at the time of treatment and 4, 7, 15 and 22 days afterwards. Further details concerning the plants at the time when the experiment was begun are summarized below: —

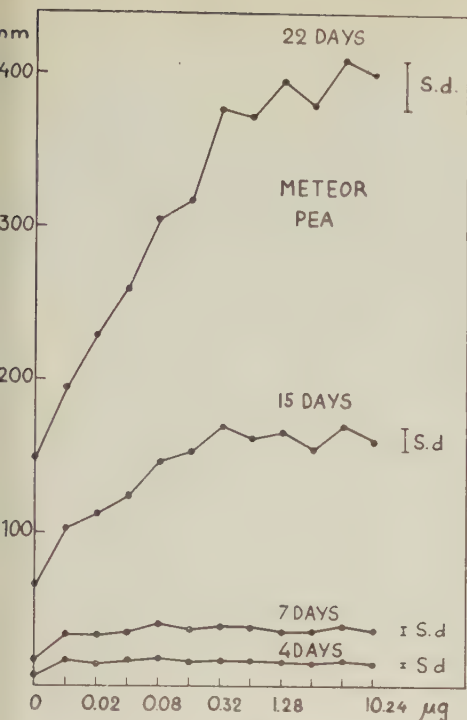


Figure 1.

Figure 1. Response of Meteor pea seedlings to single doses of gibberellic acid applied to first true leaf. — On the abscissa dose of gibberellic acid; on the ordinate mean growth increment per plant. S. d. = $S.E. \times t \times \sqrt{2}$ ($P = 1\%$).

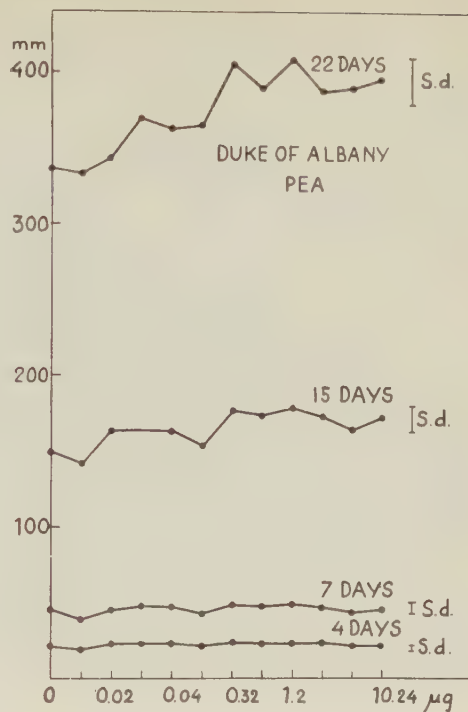


Figure 2.

Figure 2. Response of Duke of Albany pea seedlings to single doses of gibberellic acid applied to first true leaf. — For explanation see Figure 1.

Variety	Age (days)	Height ¹ (mm.)	Internodes visible
Meteor	28	20	6
Duke of Albany	24	28	6
Improved Pilot :	24	30	5

¹ above node bearing first true leaf.

The effect of gibberellic acid treatment on the growth increments of each of the three varieties is shown in Figures 1—3, the dose of gibberellic acid being plotted on a log scale. The results obtained from analysis of variance of the data are summarised in Table 1. It is convenient to consider each variety separately in the first place.

Meteor. The treatment variance was significant at all times of measure-

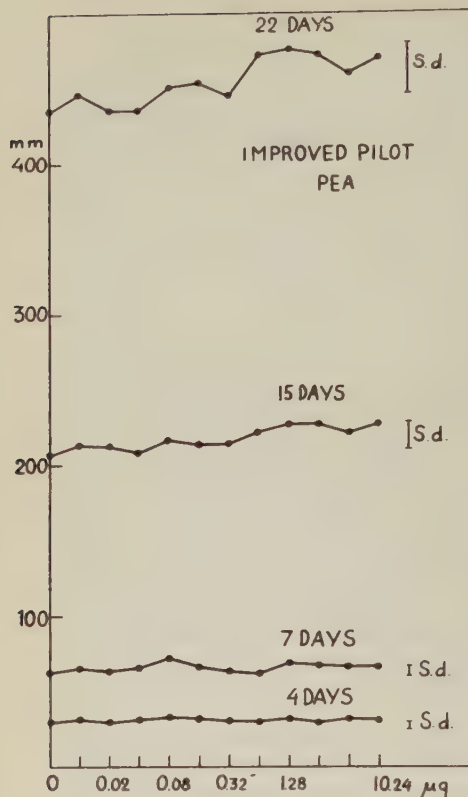


Figure 3. *Response of Improved Pilot pea seedlings to single doses of gibberellic acid to first true leaf. — For explanation see Figure 1.*

ment. Four days after treatment the lowest dose 0.01 μg .) of gibberellic acid caused a significant increase in growth increment as compared with the untreated control, but it is doubtful whether there is any significant difference in response to increased doses above this minimum. The situation was similar after 7 days. At 15 days a more definite dose-response relation

Table 1. *Gibberellic acid dose-response experiment: ratio (F) of treatment and residual error variances.*

Time	Meteor	Duke of Albany	Improved Pilot
4 days	23.8 ²	1.5	1.3
7 days	33.9 ²	1.7	1.3
15 days	90.3 ²	5.2 ²	1.9 ¹
22 days	141.6 ²	8.3 ²	7.1 ²

¹ significant at 5 % level.

² , , 1 % ,



Figure 4. *Meteor* pea seedlings 26 days after application of gibberellic acid (GA). — Reading from right to left: — no. 1, untreated; no. 2, alcohol control; no. 3, 0.01 μg . GA, and thence doses increasing in twofold steps to no. 13, 10.24 μg . GA.

could be seen, the growth increment steadily increasing with dose up to 0.32 μg . At 22 days the curve relating response to log dose was linear for doses of 0.01—0.32 μg ., a maximum response being obtained with a dose of 5.12 μg . The appearance of the plants 26 days after treatment can be seen in Figure 4.

Thus a large dose shows a progressively greater effect than a low dose as the time after treatment increases. This is reflected in the response of individual internodes (Figure 5). Internode 4, which had almost ceased extending at the time of application of gibberellic acid, showed no response to treatment. Internode 5 showed a definite response, but the lowest dose (0.01 μg .) produced a near maximal response. With each succeeding internode the greater effect of increased dose becomes more apparent, and internode 10 only reached measurable size in plants which had received doses of 0.32—10.24 μg . gibberellic acid.

Duke of Albany. Treatment variance was only significant in the measurements at 15 and 22 days. Even after 22 days the curve relating response to

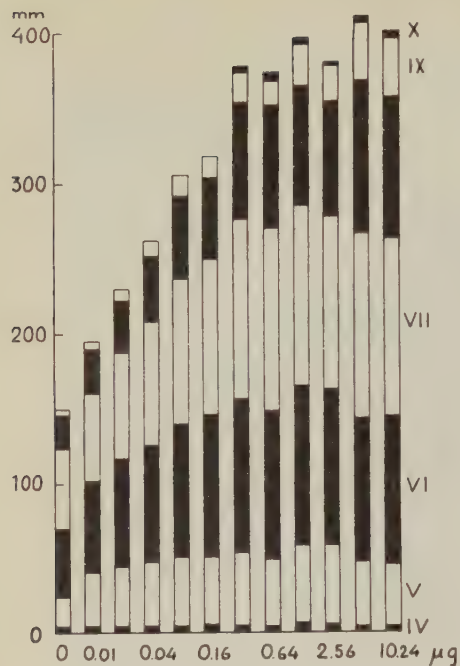


Figure 5. *Response of individual internodes of Meteor pea to single doses of gibberellic acid applied to first true leaf.* — Internodes numbered with Roman numerals. For further explanations see Figure 1.

log dose was much flatter than with the variety Meteor; in other words gibberellic acid has much less effect on the growth rate of this variety than on Meteor. Whereas after 22 days a Meteor plant which had received a dose of 5.12 μg . gibberellic acid showed a growth increment nearly 3 times that of an untreated plant, with Duke of Albany the same dose produced a growth increment only 20 % greater than the untreated control, nor was any dose significantly greater in its effect than this.

Improved Pilot showed an even smaller response than Duke of Albany.

The 22 day mean growth increment of untreated plants of these three varieties was: — Meteor, 149 mm.; Duke of Albany, 337 mm.; Improved Pilot, 436 mm. The mean growth increments of plants which had received 10.24 μg . of gibberellic acid were, respectively, 401 mm., 396 mm. and 470 mm. Two aspects of this differential response are of particular interest, *viz.* (i) the greater the growth rate of the variety, the smaller was the response to gibberellic acid; (ii) after treatment with gibberellic acid the growth rates of all three varieties were of the same order. It was considered desirable to assess the significance of these observations by an experiment with a greater number of varieties of pea.

Table 2. Mean growth increments (mm. per plant) of pea seedlings 7 days after spraying with water, aqueous indolylacetic acid (IAA₂=2 µg./ml., IAA₂₀=20 µg./ml.) or aqueous gibberellic acid (GA₁₀=10 µg./ml., GA₁₀₀=100 µg./ml.).

Variety	Initial height ¹ (mm.)	Treatments					Varietal Mean
		Water	IAA ₂	IAA ₂₀	GA ₁₀	GA ₁₀₀	
Onward	48.0	19.3	16.8	23.5	43.0	62.8	33.1
Peter Pan	55.0	28.3	24.8	32.0	57.8	67.5	42.1
Gladstone	60.3	30.0	34.0	36.3	62.3	73.3	47.2
Phenomenon	54.0	30.5	25.5	33.0	69.0	80.3	47.7
Kelvedon Wonder	55.8	31.0	26.5	33.0	69.3	80.3	48.0
Autocrat	45.5	34.5	33.5	39.0	67.0	85.8	52.0
Senator	79.8	54.0	56.3	55.0	94.0	104.3	72.7
Achievement	91.8	66.8	65.0	72.8	89.8	108.5	80.5
Duke of Albany	66.5	75.5	82.5	75.5	76.5	94.8	81.0
Gradus	105.3	78.5	83.3	82.3	91.5	99.8	87.1
Improved Pilot	108.0	119.8	124.3	119.3	120.5	138.0	124.4
Treatment mean		51.7	52.1	54.6	76.4	90.5	

¹ above node bearing first true leaf.

Significant difference ($S.E. \times t \times \sqrt{2}$) for comparison of individual means:

7.4 (P=5 %), 9.9 (P=1 %)

» » » » comparison of treatment means:

2.2 (P=5 %), 2.9 (P=1 %)

» » » » comparison of varietal means:

3.3 (P=5 %), 4.3 (P=1 %)

Differential response of pea varieties

For this experiment the eleven varieties listed in Table 2 were chosen; 14 day old plants were treated by spraying until just before run-off with water, aqueous solutions of gibberellic acid (10 or 100 µg./ml.) or indolylacetic acid (2 or 20 µg./ml.). All treatments were replicated four times. A preliminary experiment had shown that IAA produced a maximal response at 20 µg./ml.; on the other hand 100 µg./ml. gibberellic acid was probably sub-optimal. The mean growth increments after 7 days for each treatment on each variety are recorded in Table 2.

In the analysis of variance it was found that variances due to treatments and to varieties were both highly significant ($P < 1\%$) and that the interaction between treatments and varieties was also significant ($P < 1\%$). Considering first the treatment means for all varieties combined, it is clear that both gibberellic acid treatments produced a great increase in the growth increment; the lower dose of indolylacetic acid had no effect, but the 20 µg./ml. spray caused a small increase in growth increment, just reaching significance at the 1 % level. If the individual treatment means for each variety are inspected it will be seen that the slower-growing varieties showed

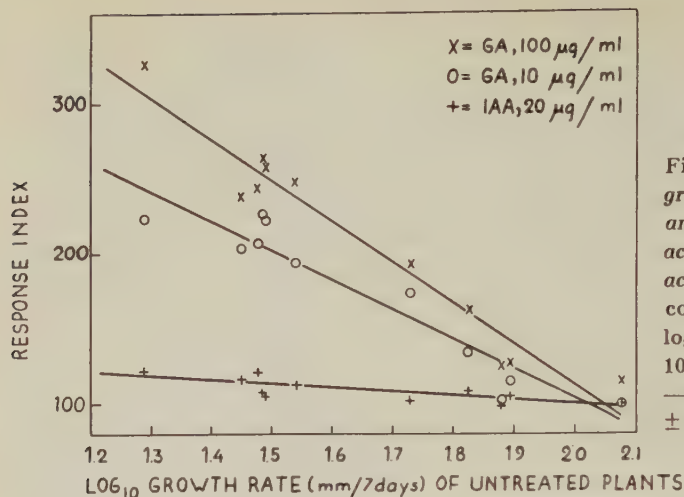


Figure 6. Relation between growth rate of pea varieties and response to gibberellic acid (GA) and indolylacetic acid (IAA) sprays. Regression coefficients of response on \log_{10} growth rate are: — GA 100, -274.4 ± 21.5 ; GA 10, -196.9 ± 21.5 ; IAA 20, -26.4 ± 6.2 .

a great response to gibberellic acid treatment, whereas the rapidly-growing varieties showed little or no response, confirming the earlier result with three varieties.

This is more clearly shown in Fig. 6. Here, a response index, calculated as follows: —

$$\frac{\text{mean growth increment of treated plants}}{\text{mean growth increment of untreated plants}} \times 100$$

has been plotted against log growth rate of the untreated plants for each variety. The varietal response index for both gibberellic acid treatments and the 20 $\mu\text{g./ml.}$ indolylacetic acid spray is linearly related to log growth rate of untreated plants, and highly significant regression lines have been fitted. The regression lines for gibberellic acid are far steeper than that for indolylacetic acid and the net effect of gibberellic acid treatment has been once again to reduce the differences in growth rate between varieties.

Discussion

Dose-response relations

The effects of doses of gibberellic acid in the range 0.01—10.24 $\mu\text{g.}$ per plant were only satisfactorily distinguished in the experiment using the variety Meteor (Figures 1 and 4). We have seen that shortly after treatment all doses in that range produced an approximately equal effect, but that as

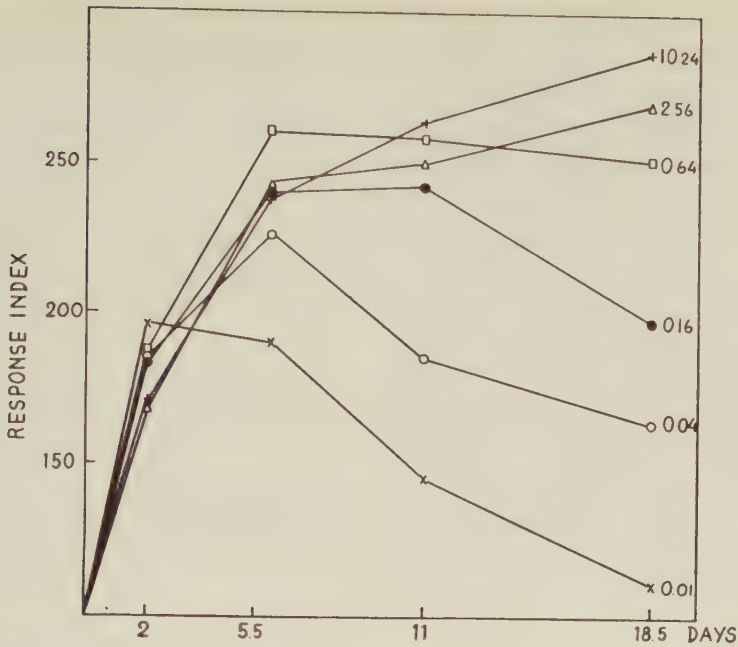


Figure 7. Growth rate of *Meteor* peas treated with gibberellic acid related to that of untreated controls. — Doses of gibberellic acid given to the right in the diagram. For derivation of response index see text.

the time after treatment increased large doses produced progressively greater growth responses than low doses. This relation can be further analysed and more fully understood if a 'response index' is derived for each treatment for the intervals between each measurement. The response indices (viz. mean daily growth rates expressed as a percentage of the growth rate of untreated plants) have been plotted against time in Fig. 7, the assumption being made that the relative growth rates measured by the response indices were reached at a point in time intermediate between each measurement. Thus measurements were made at 0, 4, 7, 15 and 22 days and the response indices attributed to 2, 5.5, 11 and 18.5 days. Alternate doses have been omitted to avoid overcrowding the figure.

Initially all doses increased growth rate to a similar extent. The growth rate of plants which received the highest dose (10.24 µg.) of gibberellic acid continued to increase in relation to the growth rate of untreated plants throughout the experiment. The relative growth rate of plants receiving lower doses after a period began to decline; thus the lowest dose (0.01 µg.)

produced its maximum effect after 2 days, the growth rate thereafter falling off until at the end of the experiment it closely approached that of untreated plants. Each progressively higher dose produced an initially more prolonged effect, reaching a greater growth rate as compared with the untreated plants, but the growth rates for each dose each fell away in turn from their appropriate maximum.

Thus, either by being used up in some growth reaction, or by degradation, or by progressive dilution in the increasing volume of tissue, or by a combination of several of these processes, the initial effect of a given dose begins to fall off with time; the greater the dose the more prolonged is its effect. However, the fact that a dose of 10.24 $\mu\text{g.}$ per plant still had an increasing effect after 18.5 days, and that a dose of 0.01 $\mu\text{g.}$ had over a period of 2 days as great an effect as the higher dose, indicates that gibberellic acid is fairly stable in the plant and is of a remarkably high degree of activity. Further experiments will be necessary to determine which of the suggested reasons for the eventual decline in effect of a single dose are in fact operative.

Differential varietal response

We have shown that there is a linear relationship between the log growth rate of a variety of pea and its 'response index' to gibberellic acid or indolylacetic acid. The varieties used (Table 2) can be roughly divided into two groups as follows: —

Vars. with low growth rate

Onward
Peter Pan
Gladstone
Phenomenon
Kelvedon Wonder
Autocrat

Vars. with high growth rate

Senator
Achievement
Duke of Albany
Gradus
Improved Pilot

All those which showed a high growth rate in our experiment may be considered to be tall varieties, with a height at maturity above 3 feet. With two exceptions those with a low growth rate are dwarf peas with a height at maturity not exceeding 2 feet. The two exceptions are Gladstone and Autocrat which though above 3 feet at maturity are late maturing varieties, in other words they have a longer period of growth than the other varieties. Thus if we consider plants with roughly the same life span it is true to say that gibberellic acid and indolylacetic acid have a greater effect on dwarf peas than on tall ones. There is a marked quantitative difference between



Figure 8. *Right, untreated Meteor pea; centre, Meteor pea treated with 10.24 μ g. gibberellic acid; left, untreated Duke of Albany pea. All 26 days old.*

the effect produced by gibberellic acid and that produced by indolylacetic acid. Though indolylacetic acid only slightly increases the growth rate of dwarf peas and has little or no effect on tall peas, it does not appreciably reduce the gap in growth rate between dwarf and tall peas. Gibberellic acid, also with little effect on tall peas, has a much greater effect on dwarf peas, so that if a sufficiently high dose is given (Figures 1—3) the growth rate of a dwarf variety (Meteor) can be made to approximate to that of tall varieties (Duke of Albany or Improved Pilot). This is clearly shown in Figure 8 where an untreated Meteor plant, a Meteor plant treated with 10.24 μ g. gibberellic acid and an untreated Duke of Albany plant are shown side by side.

Dwarf forms have arisen in many species of plant, and have been perpetuated in several cultivated plants, notably in peas, broad beans (*Vicia*) and french beans (*Phaseolus*). The dwarf plants in each case show symptoms of some form of derangement in auxin metabolism. Some earlier workers have attributed the dwarf condition to more rapid destruction of auxin (de Haan and Gorter, 1936; van Overbeek, 1938). A more recent investigation by von Abrams (1953) failed to confirm this view. He found that indolylacetic acid applied as a spray increased stem extension of a dwarf pea and somewhat decreased that of a tall pea. Our result in general confirms his, but in so far as we have used a greater number of varieties we have made the observation more precise and have demonstrated the existence

of a definite relation between response to exogenously applied indolylacetic acid and the intrinsic growth rate of a variety. Von Abrams, in further experiments, was unable to demonstrate any difference in auxin content between his tall and dwarf varieties, nor in capacity to destroy indolylacetic acid or in capacity to produce indolylacetic acid from its natural precursor tryptophane. Adding to these negative observations the fact that indolylacetic acid treatment did not increase the growth rate of his dwarf variety to anything approaching that of his tall variety, von Abrams concluded that 'auxin deficiency' in the dwarf was not the fundamental cause of dwarfism. Our experience with indolylacetic acid confirms his, but application of gibberellic acid elicited a response which virtually eliminated the distinction between tall and dwarf varieties. An understanding of the mode of action of gibberellic acid can therefore hardly fail to give us further insight into the physiological basis of the dwarf habit in peas and, conversely, the existence of the differential response between tall and dwarf peas should be of assistance in mode of action studies with gibberellic acid. We have found that there is a similar differential response between tall and dwarf varieties of broad bean (*Vicia*) and french bean (*Phaseolus*), so that the causes of dwarfism in these plants are probably the same as those acting in peas.

Summary

- (1) The growth rate of shoots of seedlings of the dwarf pea variety Meteor is significantly increased by application of 0.01 μg . gibberellic acid per plant. Higher doses produce no greater effect if measurements are made shortly (4 days) after treatment, but in longer term experiments there is a linear relation between log dose and growth response in the dose range 0.01—0.32 μg . per plant.
- (2) The growth rate of plants receiving a single dose increases relatively to that of untreated plants for a period, but eventually falls again to the growth rate level of untreated plants. The larger the dose (in the range 0.01—10.24 μg .) the longer the period of enhanced growth rate is prolonged.
- (3) Slow growing varieties of pea, e.g. dwarf varieties, respond more to application of gibberellic acid than fast growing varieties, e.g. tall varieties. Suitable doses of gibberellic acid virtually eliminate the difference in growth rate between tall and dwarf varieties. Indolylacetic acid has a qualitatively similar but much smaller effect. In an experiment

with 11 varieties of pea the response to gibberellic acid, as measured by a 'response index', was linearly and inversely related to the log growth rate of a given variety at the time of treatment.

We are indebted to Miss P. S. Hooper, Mr. I. H. Petty and Mr. R. G. Weaver for technical assistance. The gibberellic acid used in this work was prepared by our colleagues Mr. P. J. Curtis and Mr. G. L. F. Norris.

References

- von Abrams, G. J.: Auxin relations of a dwarf pea. — *Plant Physiol.* 28: 443. 1953.
- Borrow, A., Brian, P. W., Chester, V. E., Curtis, P. J., Hemming, H. G., Henehan, C., Jefferys, E. G., Lloyd, P. B., Nixon, I. S., Norris, G. L. F. & Radley, M.: Gibberellic acid, a metabolic product of the fungus *Gibberella fujikuroi*: some observations on its production and isolation. — *J. Sci. Food Agric.* 6: 340. 1955.
- Brian, P. W., Elson, G. W., Hemming, H. G. & Radley, M.: The plant-growth-promoting properties of gibberellic acid, a metabolic product of the fungus *Gibberella fujikuroi*. — *J. Sci. Food Agric.* 5: 602. 1954.
- Cross, B. E.: Gibberellic acid. Part. I. — *J. chem. Soc.* 4670. 1954.
- Curtis, P. J. & Cross, B. E.: Gibberellic acid. A new metabolite from the culture filtrates of *Gibberella fujikuroi*. — *Chem. & Ind.*, 1066. 1954.
- de Haan, I. & Gorter, C. J.: On the differences in longitudinal growth of some varieties of *Pisum sativum*. — *Rec. trav. bot. Neerl.* 33: 434. 1936.
- Mulholland, T. P. C. & Ward, G.: Gibberellic acid. Part. II. The structure and synthesis of gibberene. — *J. chem. Soc.* 4676. 1954.
- van Overbeek, J.: Auxin production in seedlings of dwarf maize. — *Plant. Physiol.* 13: 587. 1938.
- Stodola, F. H., Raper, K. B., Fennell, D. I., Conway, H. F., Sohns, V. E., Langford, C. T. & Jackson, R. W.: The microbiological production of gibberellins A and X. — *Arch. Biochem. Biophys.* 54: 240. 1955.
- Yabuta, T. & Hayashi, T.: Biochemistry of the 'bakanae' fungus (2). Isolation of gibberellin, a metabolic product of *Gibberella fujikuroi* Wr. which promotes the growth of rice seedlings. — *J. agr. chem. Soc. Japan.* 15: 257. 1939.

The Metabolism of Rumex Virus Tumors. Terminal Respiratory enzymes

By

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Introduction

Our knowledge of the terminal respiratory enzymes in plant neoplasms is extremely limited and consists of the results of several investigations made on crown-gall tumors (1). Explants of tumors induced by a specific virus on the roots of *Rumex acetosa* L. will grow in a chemically defined medium thus providing an excellent source of pathological material for studies on the metabolism of neoplastic growth. The cancerous nature of this tissue plus the presence of a known causative agent within the host cells would lead one to expect that the metabolism of Rumex virus tumors is different in some respect from that of normal tissues. This report represents part of an investigation designed to determine the differences, if any, in the metabolism of normal and tumor tissues and deals with the enzymes involved in the terminal respiration of virus tumors.

Materials and Methods

Rumex virus tumors were grown in culture on the medium of Burkholder and Nickell (2). The cultures were maintained at 22° C under continuous illumination. The methods used to transfer and prepare the tissue for respiratory studies have been described (3).

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Tumors were homogenized in a cold mortar and pestle containing acid-washed sand with an equal amount (W/V) of cold 0.05 *M* phosphate buffer at the pH indicated for each experiment. The resulting suspension was strained through cheesecloth and then centrifuged at $1000 \times g$ for ten minutes. The supernatant from this centrifugation was used directly as the source of enzymes.

Reduced cytochrome *c* was prepared by dissolving cytochrome *c* (0.5 mg/ml) in 0.03 *M* phosphate buffer, pH 7.7, and adding sodium hydrosulfite to a final concentration of 0.004 *M*. The resulting solution was aerated for ten minutes and sufficient buffer added, when necessary, to adjust to the desired optical density at 5500 Å.

Cytochrome *c* and reduced diphosphopyridine nucleotide were obtained from the Sigma Chemical Co. Pyrogallol and succinic acid were obtained from Mallinckrodt Chemical Works. 2,6 dichlorobenzenoneindophenol (Na salt), catechol, and *p*-phenylenediamine from Eastman Kodak Co., and hydrogen peroxide from Merck & Co. Ascorbic acid and tyrosine were obtained from General Biochemicals, Inc., hydroquinone from The Matheson Co., and bovin plasma albumin from Armour Laboratories.

Protocols for individual experiments are given in the text or in legends accompanying the figures.

Results

The terminal oxidases present in living systems may be classified as follows:

I. Metal-protein oxidases

A. Iron-protein oxidases, including:

1. Cytochrome oxidase
2. Catalase
3. Peroxidase

B. Copper-protein oxidases, including:

1. Polyphenol oxidase
2. Laccase
3. Ascorbic acid oxidase

II. Flavin oxidases, e.g. glycolic acid oxidase.

The present investigation is concerned with enzymes from each of these groups.

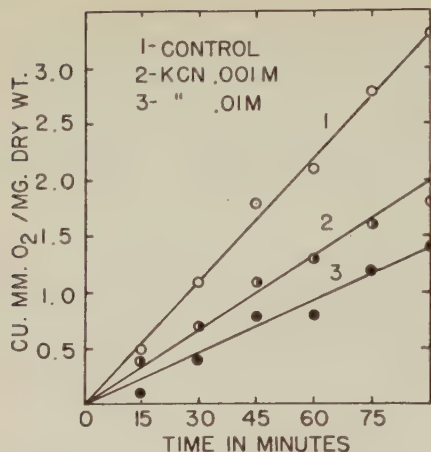


Figure 1.

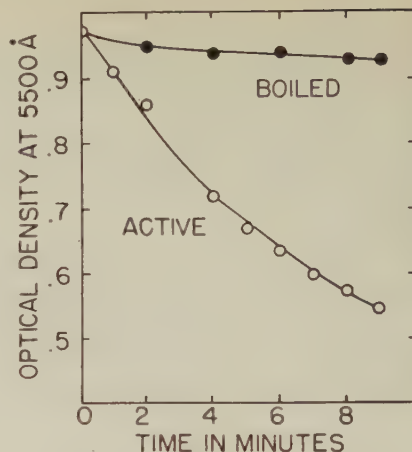


Figure 2.

Figure 1. Cyanide inhibition of the respiration of slices of *Rumex virus* tumors at 25° C. Tissue suspended in 3.0 ml 0.05 M phosphate buffer, pH 6.0, containing KCN where indicated. Center well contained 0.2 ml 20 % KOH in the absence of KCN. In vessels containing 0.001 M and 0.01 M KCN in the main compartment, the center wells contained 1.0 M and 2.0 M KCN respectively. Filter paper wicks were used in all center wells. Manometers were read every 15 mins. after a 30 min. equilibration period.

Figure 2. Cytochrome oxidase activity of an extract of *Rumex virus* tumors. Activity determined spectrophotometrically at 5500 Å by the change in optical density accompanying the oxidation of reduced cytochrome c by an enzyme preparation. Reaction mixture contained 3.0 ml reduced cytochrome c solution, pH 7.7, and 0.03 ml enzyme preparation. Boiled enzyme preparation served as a control.

Cyanide Inhibition of Respiration. Cyanide inhibition of the oxygen uptake of slices of tumor tissue is shown in figure 1. In the vessels containing cyanide in the main compartment, cyanide was also used as the CO₂ absorbent in the center well as suggested by Laties (4). Cyanide (0.001 M) inhibited respiration by about 45 % in 90 minutes and this inhibition was only slightly greater with a higher concentration of cyanide (0.01 M), suggesting that the cyanide-sensitive respiratory system was almost completely inhibited at the lower concentration of the inhibitor. The inhibition of oxygen uptake by cyanide suggests, of course, the presence of a heavy-metal mediated oxidase system. A residual cyanide respiration suggests the presence of a flavin oxidase system.

The Cytochrome System. Cytochrome oxidase and the cytochrome c reductases are the enzymes of this system involved in this study. The latter group of enzymes have been distinguished by Lardy (5) according to their

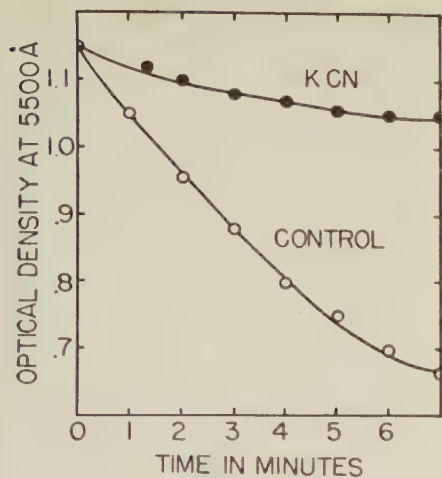


Figure 3.

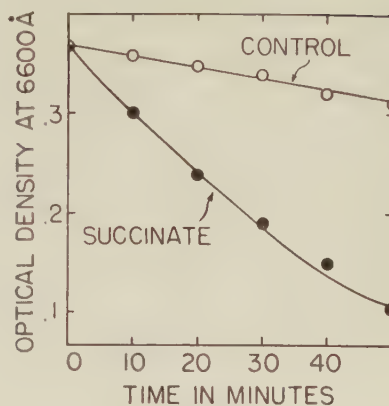


Figure 4.

Figure 3. Inhibition of cytochrome oxidase activity by 0.001 *M* KCN. Protocol same as in figure 2; KCN, 0.001 *M*.

Figure 4. Succinic dehydrogenase activity of *Rumex virus tumor* extracts. Reaction mixture in Thunberg tubes: 0.0001 *M* 2,6-dichlorobenzeneindophenol in 0.05 *M* phosphate buffer, pH 7.0, 4.0 ml; bovin plasma albumin, 0.8 mg; succinate, 1.0 *M* (or buffer), 0.1 ml; enzyme preparation, 0.5 ml. Control mixture lacked substrate.

reducing donors, e.g. succinic acid, reduced diphosphopyridine nucleotide (DPNH), and reduced triphosphopyridine nucleotide (TPNH).

Cytochrome oxidase was demonstrated spectrophotometrically in a Beckman DU spectrophotometer by following the change in optical density at 5500 Å accompanying the oxidation of reduced cytochrome *c* (Figure 2). This method has the advantage of requiring only small quantities of tissue, and is particularly desirable when the tissue under investigation is grown in culture. The tissue homogenates and the reduced cytochrome *c* solution were prepared at pH 7.7 as previously described. The cytochrome oxidase was inactivated by boiling and was inhibited by cyanide, 0.001 *M* (Figure 3).

Although the coenzyme-linked cytochrome *c* reductases and probably succinic dehydrogenase are flavin-containing enzymes, their intimate association with the cytochrome system warrants their inclusion in this section. Attempts to demonstrate the oxidation of succinate by tissue homogenates in Warburg respirometers were unsuccessful. Also, the reduction of cytochrome *c* by a succinate system was not indicated. However, succinic dehydrogenase activity was demonstrated by the anaerobic reduction of the dye, 2,6 dichlorobenzeneindophenol (Figure 4). These experiments were performed essentially as described by Price and Thimann (6). The tissue homogenate was

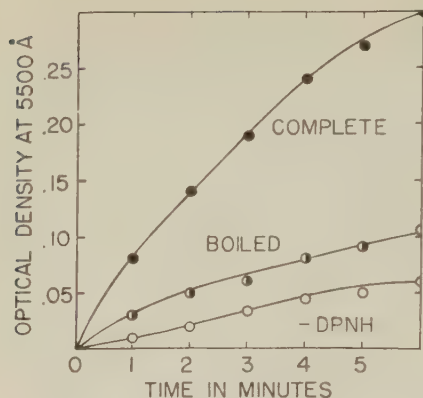


Figure 5.

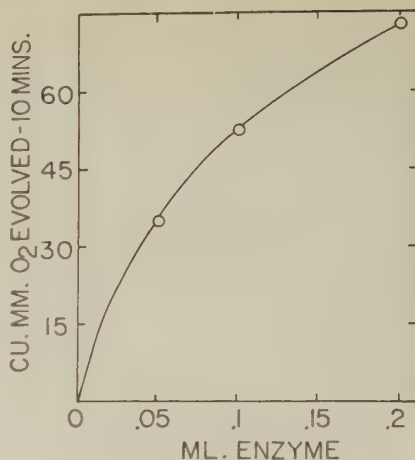


Figure 6.

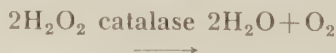
Figure 5. DPNH-linked cytochrome *c* reductase activity of *Rumex virus* tumor extracts. Reaction mixture contained 2.7 ml of 0.05 *M* phosphate buffer, pH 7.0, containing 0.5 mg/ml cytochrome *c*; 0.1 ml KCN (0.1 *M*); 0.1 ml DPNH (6.0 mg./ml.); 0.1 ml enzyme preparation. Controls consisted of a complete mixture containing boiled enzyme and another lacking DPNH.

Figure 6. Catalase activity of an extract of *Rumex virus* tumors. Oxygen evolution measured at 25° C. in Warburg respirometers, total fluid volume 1.2 ml. Vessels contained indicated amount of enzyme plus sufficient phosphate buffer, 0.05 *M*, pH 7.1, to make 1.0 ml. Sidearm contained 0.1 ml of 0.3 *M* H₂O₂ or water. Contents of sidearm tipped in at 0 time. Center well contained 0.1 ml 20 % KOH and filter paper wick.

prepared by grinding the tumors in 0.2 *M* sucrose in 0.03 *M* phosphate buffer, pH 7.0. The reaction was carried out in Thunberg tubes *in vacuo* and the reduction of the dye was measured in a Klett-Summerson colorimeter. Reduction of the dye in the absence of substrate was negligible.

A DPNH-linked cytochrome *c* reductase was demonstrated (Figure 5) by measuring the reduction of cytochrome *c* in the presence of cyanide. No attempt was made to demonstrate a similar system involving TPNH.

Catalase and Peroxidase. Both catalase and peroxidase are iron-proteins which react with hydrogen peroxide as follows:



where R could be many organic compounds (7).

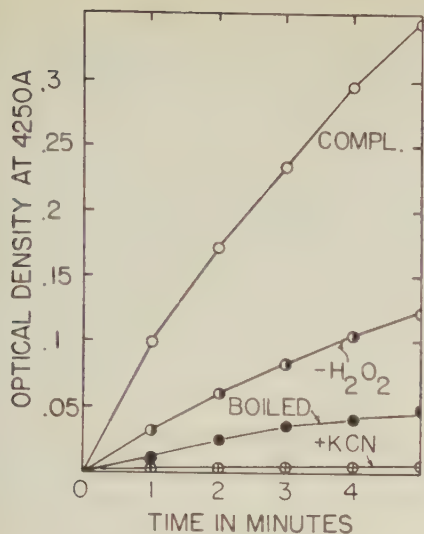


Figure 7.

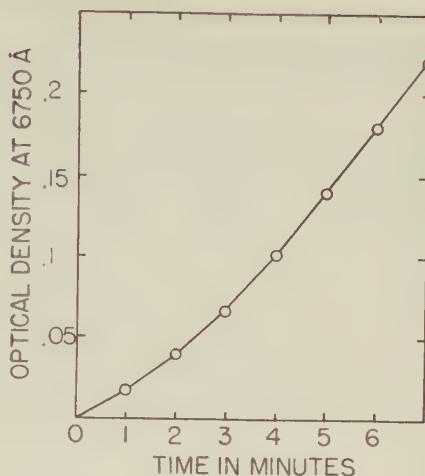


Figure 8.

Figure 7. *Peroxidase activity of Rumex virus tumor extracts.* Oxidation of pyrogallol was measured spectrophotometrically at 4250 Å. Complete reaction mixture contained phosphate buffer, 0.05 M, pH 7.1, 2.8 ml; pyrogallol (5 %), 0.1 ml; enzyme preparation, 0.01 ml; H₂O₂ (1 %), 0.1 ml; KCN 0.001 M when included. A complete mixture containing boiled enzyme and one lacking H₂O₂ served as controls.

Figure 8. *Oxidation of catechol by peroxidase system in extract of Rumex virus tumors.* Reaction mixture contained phosphate buffer, 0.05 M, pH 7.1, 2.55 ml; catechol (30 mg/ml), 0.2 ml; enzyme preparation, 0.15 ml; H₂O₂ (1 %), 0.1 ml. Activity with boiled enzyme preparation or in the absence of hydrogen peroxide was negligible.

Catalase activity in tumor homogenates was demonstrated manometrically by measuring the evolution of oxygen in the presence of enzyme and hydrogen peroxide. Figure 6 shows the amount of oxygen evolved at several enzyme concentrations. There was no measurable evolution of oxygen when the enzyme preparation was boiled or in the absence of hydrogen peroxide.

Peroxidase. Peroxidase activity was determined by the oxidation of pyrogallol and catechol in the presence of hydrogen peroxide. Peroxidase activity based on the oxidation of pyrogallol to purpurogallin is shown in figure 7. The oxidation of pyrogallol was followed at 4250 Å in a Beckman spectrophotometer. The results are expressed as changes in optical density; no attempt was made to convert these figures to mg. purpurogallin. Oxidation of pyrogallol was completely inhibited by 0.001 M cyanide. The oxidation of catechol by a peroxidase system was also shown (Figure 8). Oxidation of catechol was followed at 6750 Å and there was no measurable change in

optical density in the absence of hydrogen peroxide or if the enzyme preparation was boiled. This method is essentially the same as that used by Waygood (8) to indicate peroxidase activity in wheat.

Copper-protein and flavin oxidases. All attempts to demonstrate the presence of the copper oxidases — polyphenol oxidase, laccase, and ascorbic acid oxidase — were unsuccessful. A number of attempts were made to show ascorbic acid oxidation in Warburg respirometers, and by the spectrophotometric method described by Racker (9). No ascorbic acid oxidase activity was indicated. It would also appear that polyphenol oxidase and laccase activity are lacking in these tumors. It was stated in a previous section that catechol was not oxidized in the absence of hydrogen peroxide. Since some polyphenol oxidase systems will not oxidize catechol, but will oxidize related compounds (10), other substrates were utilized. There was no oxidation of tyrosine, p-phenylenediamine, and hydroquinone. In addition, tissue homogenates showed no evidence of darkening upon standing.

The presence of a residual cyanide respiration suggests that a flavin oxidase system is present, but attempts to demonstrate glycolic acid dehydrogenase were unsuccessful. No other studies on the possible presence of a flavin oxidase system were conducted.

Discussion

On the basis of the evidence presented in this paper, it appears that the iron-containing enzymes are the most important, if not the only enzymes involved in terminal oxidation in *Rumex* virus tumors. It is possible that a flavin oxidase system is present, although the only evidence to support this view is the presence of a residual cyanide respiration. It should be noted that the degree of cyanide inhibition of respiration may depend upon the method used to measure oxygen uptake (11). However, since the oxygen uptake in the presence of 0.01 M KCN was considerable, it would appear that there is a real cyanide insensitive respiration. Although the question of a flavin oxidase system is still open, it appears that a copper oxidase system is completely lacking.

A cyanide-sensitive cytochrome oxidase system as well as two cytochrome c reductases, e.g. DPNH and succinate, are present. Reduction of cytochrome c by the succinate system could not be demonstrated but the presence of succinic dehydrogenase was indicated by dye reduction. It is believed that the reduction of cytochrome c by a succinate system is not direct (12) but

that a carrier or a special enzyme may be interposed between succinic dehydrogenase and the cytochrome system (13).

It is possible that these intermediates are inactivated during the preparation of the enzyme. The importance of the reductases in terminal oxidation is impossible to evaluate at this time, but it would seem that the cytochrome system occupies the most important position in the respiratory pattern. However, both catalase and peroxidase are present although the physiological importance of these enzymes needs further investigation. It is believed that increasing knowledge of the role of these enzymes may link them directly with respiration. Cheng (11) has pointed out that amino acids produce hydrogen peroxide on oxidation and this hydrogen peroxide can be utilized by catalase and peroxidase. This author further states that the extremely low concentration of peroxide *in vivo*, a much lower concentration than that of oxygen, argues against the participation of peroxidase in normal respiration.

The information available concerning the enzymatic processes of animal tissues, both normal and neoplastic, is considerable, although incomplete. Although a good deal of information is available concerning plant enzymology, few investigations on the enzyme systems of plant tumors have been conducted. Evaluation of the data presented here awaits further studies on control tissues grown under identical conditions. The chief difficulty in conducting comparative physiological studies with *Rumex* virus tumors is the inability to grow normal host tissue in culture. However, further efforts should be directed toward this end. In addition, tumors of different types can be induced in a number of plants from which normal and neoplastic explants can be grown in culture. A comparative study of the enzymatic processes of such tissues would be most welcome.

Summary

A survey was made of the terminal respiratory enzymes in *Rumex* virus tumor tissue grown in culture.

The oxygen uptake of slices of *Rumex* virus tumors was inhibited about 45 per cent by 0.001 *M* cyanide. This inhibition suggested the presence of a heavy-metal mediated oxidase system.

Cytochrome oxidase, DPNH-linked cytochrome *c* reductase, and succinic dehydrogenase were found to be present.

Catalase and peroxidase activities were also demonstrated.

The copper-protein oxidases — polyphenol oxidase, laccase, and ascorbic acid oxidase, were absent in this tissue.

Although a residual cyanide respiration suggested the presence of a flavin oxidase, attempts to show glycolic acid dehydrogenase activity were unsuccessful.

References

1. Braun, A. C.: The physiology of plant tumors. — *Ann. Rev. Plant Physiol.* 5: 133. 1954.
2. Burkholder, P. R. & Nickell, L. G.: Atypical growth of plants. I. Cultivation of virus tumors of *Rumex* on nutrient agar. — *Bot. Gaz.* 110: 426. 1949.
3. Gentile, A. C. & Naylor, A. W.: The metabolism of *Rumex* virus tumors. Respiration of tissue slices. — *Archiv. Biochem. Biophys.* (In Press).
4. Laties, G. G.: Limitations of the use of potassium hydroxide-potassium cyanide mixtures in manometric studies. — *Jour. Biol. Chem.* 177: 969. 1949.
5. Lardy, H. A., Ed.: *Respiratory Enzymes*. Pp. 1—290. — Burgess Publ. Co., Minneapolis, Minn. 1949.
6. Price, C. A. & Thimann, K. V.: The estimation of dehydrogenases in plant tissue. — *Plant Physiol.* 29: 113. 1954.
7. Theorell, H.: The Iron-Containing Enzymes B. Catalases and Peroxidases. »Hydroperoxidases». — In »The Enzymes» ed. by J. B. Sumner and K. Myrbäck. Vol. II, Part I. Pp. 397—427. 1951.
8. Waygood, E. R.: Physiological and biochemical studies in plant metabolism. II. Respiratory enzymes in wheat. — *Can. Jour. Resch. Sect. C.* 28: 7. 1950.
9. Racker, E.: Spectrophotometric measurements of the metabolic formation and degradation of thiol esters and enediol compounds. — *Biochim. Biophys. Acta* 9: 577. 1952.
10. Keilin, D. & Mann, T.: Polyphenol oxidase. Purification, nature and properties. — *Proc. Royal Soc. London, Ser. B.* 125: 187. 1938.
11. Cheng, S. C.: The terminal respiratory enzymes in *Neurospora tetrasperma*. *Plant Physiol.* 29: 458. 1954.
12. Keilin, D. & Hartree, E. F.: Succinic dehydrogenase-cytochrome system of cells. Intracellular respiratory system catalyzing aerobic oxidation of succinic acid. — *Proc. Royal Soc. London, Ser. B.* 129: 277. 1940.
13. Schlenk, F.: Succinic Dehydrogenase. — In »The Enzymes» ed. by J. B. Sumner and K. Myrbäck. Vol. II, Part I. Pp. 316—28. 1951.

Studies on Growth and Inhibition of *Candida albicans*

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Introduction

During the last years the yeast fungus *Candida albicans* has gained great importance as a human pathogen. This is especially due to the use of certain antibiotics which, by disturbing the balance between the microorganisms, in certain cases have given rise to infections due to overgrowth of *C. albicans*. The problem as to in which way such an infection can be eliminated has become important. In this connection the demand for special *Candida* research was discussed. This Kligman (1952) raised the question of whether these fungus infections, arising after antibiotic therapy, were of secondary importance, while the disturbed vitamin balance and the appearance of resistant staphylococci and streptococci were regarded as the primary problem. Later on, however, it became evident, through the increasing number of *Candida* infections, that a direct *Candida* therapy was necessary.

The present investigation deals with the problems mentioned above. The original intention was to find an antimetabolite capable of inhibiting the life processes of the fungus by investigating the demands of fundamentally important substances as well as the requirements of vitamins and trace elements. In this connection many substances were tested for fungistatic action. Later on, however, it was found necessary to test even substances without a known action mechanism and without connection to the growth conditions of the fungus.

C. albicans, which has a yeast phase and a mycelial phase of growth depending on the culture conditions, was investigated in this study entirely

in its yeast phase, which is primarily found on media containing glucose. Nickerson and Mankowski (1953) have shown that media rich in glucose and phosphate as well as large amounts of -SH-groups (e.g., cystein), allow yeast cells to develop while mycelial formation is entirely suppressed.

The greater part of the investigation has been performed with one strain of *C. albicans* (originally isolated from a human case of candidiasis) obtained through the courtesy of Å. Nordén, M. D., Medical Clinic, Lund, who also suggested the problem.

Methods

Stock cultures of *C. albicans* were obtained by growing the fungus at 37° C on Sabouraud's glucose-agar containing 4 per cent glucose, 1 per cent peptone and 1.5 per cent agar.

The greater part of the investigation was performed as growth experiments either with shaking cultures or with the aid of Warburg technique. In both cases a simple synthetic glucose-asparagine medium was used as the basic medium. This medium was composed in the following way: Glucose 20.0, asparagine 2.0, KH_2PO_4 1.5, $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$ 0.5 grams per litre, and biotin 0.1 microgram per litre. The following metals were added in trace amounts: Fe^{3+} 0.20, Zn 0.18, Mn 0.02 ppm. The pH of the medium was adjusted to 5.5—5.7 by the addition of NaOH before sterilizing.

These two substrates permit rapid growth of the yeast phase of the fungus and exclude mycelial growth entirely or almost entirely.

All chemicals used were standard preparations with the highest degree of purity. Glass-distilled water was used throughout the investigation. All glassware (Pyrex) was treated with dichromate cleaning solution before rinsing in tapwater and double-distilled water.

Growth experiments were carried out mostly in 200 ml Erlenmeyer flasks containing 40 ml liquid medium. The flasks were twice sterilized by steaming 25 minutes with an interval of 22—24 hours. Agar substrates were autoclaved 15 minutes at 120° C, and pipettes and other glassware were sterilized in air 150—160° C for two hours.

The culture flasks were placed on a shaking table with 100—120 reciprocal movements per minute to keep them well aerated. The flasks were kept at 25° C in darkness for at least 4 days. The results were read by weighing the dried cells after autoclaving (because of the pathogenicity of the fungus), centrifuging, and washing. Weighing was performed directly in the centrifuge tubes employed.

In growth experiments in Warburg apparatus the same liquid medium as above was used. Every flask was furnished with 2.0 ml of the basic medium, 0.5 ml of a cell suspension, and water and other substances to give a volume of 3.3 ml per flask. Two tenth ml 10 per cent KOH was given to the centre cup. The temperature was kept at 25° C as in the shaking cultures and the oscillation rate was about 110 movements per minute. The oxygen uptake was read every half an hour during the entire experimental period which always was 6 hours.

The shaking cultures as well as the Warburg flasks were inoculated with a suspension of yeast cells which was obtained by washing cells either in the basic medium (except biotin) or in water, centrifuging and resuspending. The turbidity of the sus-

pension was adjusted in a Klett-Summerson photoelectric colorimeter, using a blue filter No. 40 (400—465 m μ), to a reading of 10—100, corresponding to a cell density of about $5 \cdot 10^6$ — $5 \cdot 10^7$ cells per ml. Every 40 ml flask was inoculated with 0.5 ml of this suspension, giving an initial cell density of about $6 \cdot 10^4$ — $6 \cdot 10^5$ cells per ml. In Warburg experiments this initial cell density was about 10^6 — 10^7 cells per ml, corresponding to a dry weight of about 0.1 mg per Warburg flask.

During the earlier part of the investigation difficulties were encountered in obtaining reproducible results. Thus sometimes growth was very vigorous even in controls without added biotin, while occasionally growth was very meagre, irrespective of biotin concentrations. Later on it became evident that these differences, at least to the greater part, were due to differences in the inoculation material. During the course of the work, therefore, several ways of culturing a material for inoculation were tried. The following four methods were used:

- 1) Suspending cells from two-day cultures on glucose-agar in the basic medium (except biotin) or in water (»agar cells«).
- 2) Shaking »agar cells« in tapwater or distilled water on the shaking table for 1—2 days before inoculation (»starved cells«).
- 3) Subcultivating »agar cells« once or twice in the synthetic medium, using cells grown in this medium as inoculate.
- 4) Growing cells in a so-called chemostat to obtain an inoculation material as homogeneous as possible (»chemostat cells«).

By using the first three methods it was not possible to get rid of these variations in growth. This may at least in part depend on the age of the agar medium, on the age of the inoculation cells themselves, which was especially true in the third method, or, which is not quite excluded, on some uncontrollable differences in the media.

The third method, however, was shown to give results far superior to the others. Cells of this kind never gave these large differences mentioned above, being very homogeneous at every inoculation period.

During recent years several papers on the continuous culture of microorganisms have been published. Thus Novick and Szilard (1950) described an apparatus called a chemostat convenient for the genetic study of bacteria, and Monod (1950) another type of apparatus for the continuous culture of bacteria. Most often this method of culturing microorganisms under controlled conditions has been used for bacteria. In a few cases, however, even fungi have been grown in this way. Thus B. von Hofsten, A. von Hofsten, and Fries (1953), using a modification of Monod's apparatus, cultured *Ophiostoma multiannulatum* mainly in its conidial state.

In the continuous culture of microorganisms the growth rate is kept constant and less than the maximal growth rate. This low and constant growth rate is achieved by keeping one factor of the medium constituents at a low limiting level. Under these circumstances the growth rate is proportional to the concentration of this factor. The growth rate is then further varied with the rate of addition of the nutrient medium. The theoretical background for the continuous culture has been given by Novick and Szilard (1950 a) and Monod (1950).

For the continuous culture of *C. albicans* in its yeast phase a chemostat of the Novick-Szilard type was used. As the growth conditions of yeast cells correspond very closely to those of bacteria it was possible to use this sort of apparatus. The apparatus had been slightly modified by Dr. N. Fries (Institute of Physiological

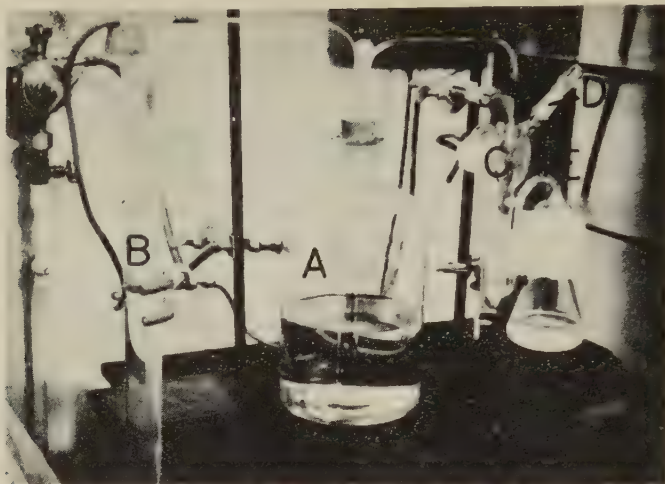


Figure 1. *The apparatus for continuous culture.* A: reservoir for the medium, B: air pressure regulator, C: culture vessel, D: inoculating port, E: outlet capillary tube, F: air-bubbling capillary tube.

Botany, Uppsala) and only a few additional modifications were needed for the culture of *C. albicans*. Thus it was found that the *Candida* cells with a mean diameter of about $5\ \mu$ showed a tendency of sinking to the bottom of the outlet capillary tube F (Figures 1 and 2) where the stirring effect of the air bubbling through the tube F is not very pronounced. This trouble, however, was eliminated by choosing a larger capillary tube and by making it rounded as in the figure instead of square.

The culture medium for the continuous culture had the same composition as the synthetic medium used in growth experiments with the exception of the biotin concentration which was lowered to $0.02\ \mu\text{g}$ per litre in order to limit growth. The transfer of the medium in the reservoir A to the culture vessel C through a capillary tube was kept at a constant rate by means of air pressure, as described by Novick and Szilard (1950). — Homogenation of the cell suspension in the culture vessel was obtained by bubbling sterile air through the solution (capillary tube F).

In this chemostat, which was inoculated through the tube D, a cell density in the culture vessel of about $5 \cdot 10^7$ cells per ml was obtained with a flow rate of 2 drops per minute. After washing, these cells were used as inoculation material. For inoculation the cells were taken directly from the culture vessel. The culture medium was then adjusted to the same volume and equilibrium was established before the next inoculation.

In this way a cell material as homogeneous as possible was obtained. Since the cells are in the phase of active growth the number of dead cells is highly reduced. The advantage of using such cells as inoculation material over the methods earlier employed is obvious. It was also very soon found that the large differences in growth results from time to time disappeared and that remaining variations could be ascribed to other factors (e.g., constituents of the medium).

In the later part of the investigation, where also Warburg technique was used in growth and inhibition experiments, it was of importance to use cells in active growth. In this respect the »chemostat cells», which were consistently very homogeneous, showed great advantage over »agar cells». In Table 1 the oxygen uptake of the same amount of »chemostat cells» and »agar cells» is measured after an experimental

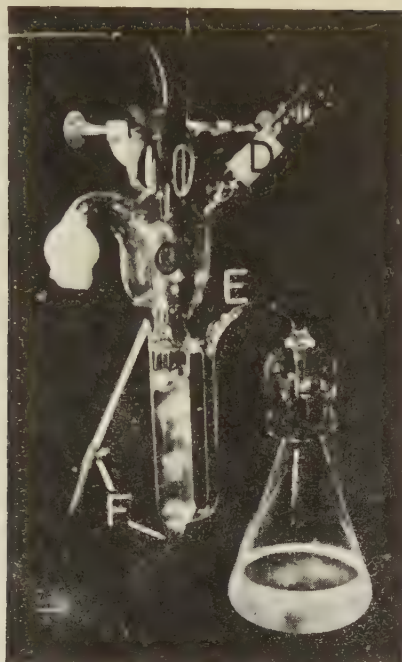


Figure 2. Detail of the chemostat arrangement. For explanations see Figure 1.

period of 6 hours. There is a marked difference between these cells, the »chemostat cells» being homogeneous and in their active growth state. A reduced number of living cells among the »agar cells», however, is not excluded. By using »chemostat cells» in growth experiments in Warburg apparatus it was possible to obtain clear-cut results even with a relatively small inoculum and within reasonable time.

Table 1. Oxygen uptake by the same amount of »chemostat cells» and »agar cells» respectively during 6 hours at 25° C. The figures represent the O₂ uptake in μ l in duplicate experiments.

Cell material	
»Chemostat cells»	»Agar cells»
86.4	25.0
78.1	32.1

Results

1. The requirements of growth substances and trace elements by *C. albicans*

The growth factors for yeasts have been investigated several times. Thus Burkholder (1943) in a study of several yeasts, about ten of which were

Table 2. *Effect of additions of the following growth factors to the synthetic medium: thiamin 200, pyridoxine 200, Ca-pantothenate 200, nicotinic acid 200, biotin 0.2, and inositol 1000 µg per litre. The figures represent dry weights in mg of shaking cultures in duplicate or triplicate flasks after an experimental period of 10 days.*

Additions of growth factors						
None	thiamin pyridoxine	pyridoxine	thiamin pyridoxine	pyridoxine	thiamin, biotin	
	Ca-pantothenate nicotinic acid biotin inositol	Ca-pantothenate nicotinic acid biotin inositol	Ca-pantothenate nicotinic acid inositol	Ca-pantothenate nicotinic acid inositol		
11	127	139	16	14	13	153
10	123	147	13	14	13	149
	128	143	12	17		

Candida species, found that all these species required biotin and that some of them also had a demand for thiamin. In similar experiments with the strain of *C. albicans* most often used in this investigation the following growth factors were tested in shaking cultures: thiamin 200, pyridoxine 200, Ca-pantothenate 200, nicotinic acid 200, biotin 0.2, and inositol 1000 µg per litre. Only biotin was shown to have any effect which is seen from Table 2. — An amount of biotin of 0.1 µg per litre was shown to be sufficient for continuous growth under these circumstances.

The requirements of trace elements of *C. albicans* were tested in a series of experiments. The medium used here was the above-mentioned glucose-asparagine medium either freed from trace metals before adding the metals under investigation or without such previous treatment. Two different methods of removing trace elements were used, the one introduced by Lilly and Leonian (1945) using charcoal and a pH of 8, the other was the method of Waring and Werkman (1943) with treatment of the solutions with 8-hydroxyquinoline and subsequent washing with chloroform.

The results from the various experiments with different treatment of the substrate are in good agreement with each other and may be summarized in the following way:

Fe showed the most pronounced enhancing effect on the growth of *C. albicans*, further strengthened by addition of Zn. A certain antagonism between Fe and Mn was observed, this antagonism being counteracted by adding Zn. Any direct growth-stimulating effect of Mn was never noticed under these conditions. Also additions of Ca, Cu, and Mo in varying concentrations were ineffective in these experiments. The following metals in the concentrations stated were also ineffective: B 0.02, Li 0.02, Be 0.10, Al 0.20, Sr 0.05, Tl 0.05, Cr 0.10, Co 0.10, Ni 0.05, Pb 0.05, and Ag 0.05 ppm (a slight inhibitory effect was noticed with Ag in this concentration).

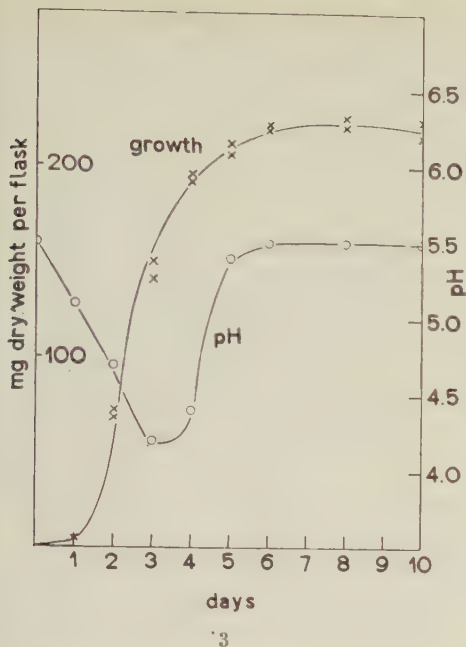


Figure 3. Growth and pH conditions in the unbuffered liquid medium during an experimental period of 10 days.

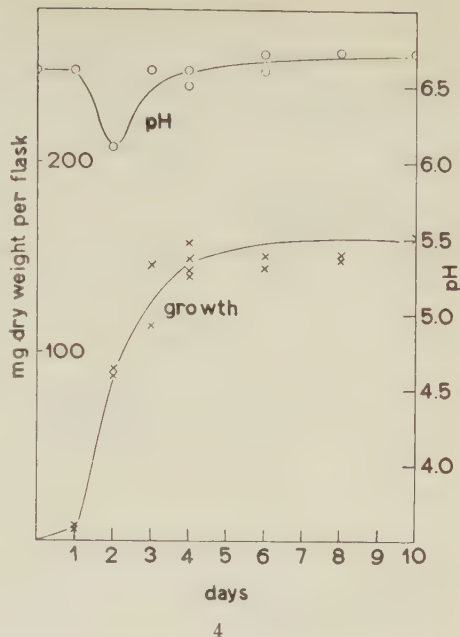


Figure 4. Growth and pH conditions in a medium buffered with $1/50$ M KH_2PO_4 and $1/50$ M Na_2HPO_4 .

The composition and the amounts of trace elements in the liquid medium used in the subsequent experiments were founded on these preliminary experiments. According to these results the following gave maximal effect: Fe 0.20, Zn 0.18, and Mn 0.02 ppm.

2. Growth and pH conditions in shaking cultures

The synthetic medium containing 20 g glucose and $0.1 \mu\text{g}$ biotin per litre allows continuous growth for about 5 days, whereupon growth stops or increases only inconsiderably which is shown in Figure 3. Under these circumstances a dry weight of about 200 mg is obtained. The glucose concentration is the limiting factor here which is seen in Figure 5, showing the connection between glucose concentration in the medium and dry weight of the fungus. The glucose determinations were performed according to Philipson (1943).

From Figure 5 it is evident that the glucose is practically used up at a dry weight of about 150 mg. This value of the dry weight is reached after 3–4

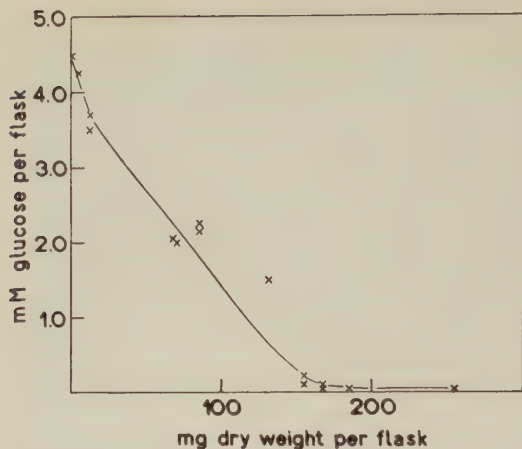


Figure 5. The connection between glucose concentration in the medium and dry weight of the fungus.

days (Figure 3), while the growth still continues for another one or two days. Therefore it was found convenient in growth experiments, where promoting or inhibiting agents were investigated, to conclude the experiment after 4 days cultivation, when the culture was still growing.

The medium employed contained only primary phosphate and, for this reason, it had only a slight buffer capacity. Therefore the pH which was adjusted to an initial value of 5.5—5.7 varies considerably during growth as is shown in Figure 3. It is interesting to notice how the pH drops from 5.5 to about 4, the dry weight at the same time increasing to about 150 mg, i.e., as long as glucose is still present in the solution. After that point the pH rapidly increases to its original value where it remains constant during the rest of the experimental period.

In order to see whether these large pH variations had any considerable retarding effect on growth, experiments were made with a medium which was strongly buffered with phosphate, 1/50 M KH_2PO_4 and 1/50 M Na_2HPO_4 , giving a pH of 6.8. The variations in pH, of course, were not as accentuated as with the unbuffered medium, although a change of about one pH unit was observed during growth (see Figure 4).

If the total growth of the fungus in the buffered medium is compared with that in the less buffered one (Figures 4 and 3), as reflected in the dry weights, no increase of growth is observed in the medium with more constant pH. On the contrary, a slight decrease in growth is noticed in this case. The economical utilization of the medium components, therefore, does not seem to be retarded even by the great changes of pH during growth.

Table 3. *The inhibiting action of different compounds.*

Substance	Lowest concentration for total inhibition	Remarks	
Desthiobiotin	—	Equally stimulative as biotin itself.	
Auxins and related compounds:			
1,2,3,4,5-pentachlorophenoxyisobutyric acid ..	$5 \cdot 10^{-4} M$		
2,4,6-trichlorophenoxyisobutyric acid	$> 5 \cdot 10^{-4} M$		
4-chlorophenoxyisobutyric acid			
4-bromophenoxyisobutyric acid	$> 10^{-3} M$		
4-iodophenoxyisobutyric acid			
α -phenoxypropionic acid	$> 10^{-3} M$		
4-chlorophenoxypropionic acid			
2,4-dichlorophenoxypropionic acid			
2,4-dichlorophenoxyacetic acid		} No effect in $10^{-5} M$	
2,4-dichlorophenylsulphoxyacetic acid			
4-chlorophenoxy-caproic acid			
diamylacetic acid	$5 \cdot 10^{-4} M$		
4-aminophenoxyisobutyric acid		} No effect in $10^{-4} M$	
4-nitrophenoxyisobutyric acid			
Gramine		} No effect in $10^{-5} M$	
Skatole			
Coumarin derivatives:			
Coumarin		} No effect in $5 \cdot 10^{-5} M$. Besides <i>C. albicans</i> also <i>Aspergillus niger</i> was tested.	
Daphnetin			
Methyl-daphnetin			
Umbelliferone			
Ethylisothiocyanate	$\sim 10^{-2} M$	Added to the medium after sterilization.	
Quaternary ammonium compounds:			
Septin (Pharmacia)	} 0.001 per cent	In tests with <i>Rhodotorula</i> , <i>Mucor</i> , and <i>Aspergillus</i> species 0.01 per cent was needed.	
Cetylpyridinium chloride			
Cetaulon			
Heart-wood substances:			
Pinosylvin	} $5 \cdot 10^{-4} M$		
Pinosylvin-monomethylether			
Selenium (as sodium selenate)		Total inhibition was found at $8 \cdot 10^{-4} M$.	
8-hydroxyquinoline	$3 \cdot 10^{-5} M$		
8-hydroxyquinoline sulphonic acid ¹		No inhibition in $10^{-3} M$	
Monoiodo-8-hydroxyquinoline sulphonic acid ¹ ..		No inhibition in $5 \cdot 10^{-4} M$	
Maleic hydrazide ¹		No inhibition in $10^{-3} M$	
2,2'-dihydroxy-5,5'-dichlorodiphenylsulphide ¹ ..	$3.5 \cdot 10^{-5} M$		
1,2,3,4-tetrahydrofluorenone (THF) ¹	$10^{-4} M$		

¹ Generously placed at my disposal by AB. Pharmacia, Uppsala.

3. Inhibition of growth by means of several different substances

In the search for an agent capable of inhibiting growth many different substances were tested. Several of these substances had some connection to the metabolism of the fungus, while others were chosen more or less at random. All agents were tested in shaking cultures, the substances generally being added to the medium before sterilization. The results, often negative, are given here only summarily (Table 3).

As is seen from the table most of these substances cause total inhibition of growth only in rather high concentrations. With reference to the medical aspect of the problem, therefore, any further investigations were in most cases not considered valuable. In one case, however, (8-hydroxyquinoline), the effect was so marked that the action mechanism was investigated more in detail.

4. Inhibition of growth by 8-hydroxyquinoline (oxine)

a) Use of oxine as chelating substance and as antimicrobial agent

Oxine is frequently used because of its ability to bind metals, thus removing heavy metals from nutrient solutions (Waring and Werkman 1943). The inhibiting action of oxine on *C. albicans* was detected by employing this method, growth being much less in one single experiment treated with oxine to remove trace elements. In this case the oxine was not totally removed because of insufficient washing with pure chloroform.

Even as a disinfectant oxine has long been used, although the action mechanism has been investigated only in recent years. Zentmeyer (1943, 1944), inhibiting growth of *Fusarium*, *Ceratostomella*, and *Penicillium* by oxine, believed this effect to be due to a pure trace element deficiency, because the inhibition was removed when Zn was added in excess. Also Albert et al. (1947) showed that the antibacterial action of oxine was connected with its metal-binding power by detecting that from the 7 isomeric monohydroxyquinolines only the compound with the OH-group in 8-position was capable of chelating action and, at the same time, antimicrobial action. Chelating action alone, however, did not secure antibacterial action which was shown by investigating several other types of chelating substances (Albert et al. 1947, 1954).

It has been shown in several investigations that an oxine inhibition may be counteracted by addition of an excess of metals (cf. Zentmeyer 1943, 1944). In this case it is significant how different metals are reported to counteract the inhibition in different organisms. Thus Alberti et al. (1947) and Rubbo et al. (1950) mention that Co neutralizes the inhibition on Gram-positive organisms but Fe and Zn on Gram-negative organisms. This action of Co is

also reported by Schuler and Meyer (1950) for *Bact. coli* and by Sorkin et al. (1951) concerning the tuberculostatic action of oxine. A corresponding counteraction with Mn (and to a lesser degree also with Co) has been pointed out by Gale (1949) for the oxine inhibition of glutamic acid accumulation in *Staphylococcus aureus*. Anderson and Swaby (1951), on the other hand, could not destroy the inhibiting action on *Aspergillus niger* with Co, Zn, Mn, Fe, or Cu, but partly with Mo.

It is further known that addition of Cu increases the inhibiting action of oxine (e.g., Sorkin et al. 1951). Rubbo et al. (1950) showed that if all traces of Fe and Cu were removed from the solution oxine lost its antibacterial action, thereby pointing out that the cause of oxine action was to be sought in the oxine-metal-complex.

The present investigation of oxine action on *C. albicans* is partly a repetition and a confirmation of results earlier obtained on other organisms, partly an attempt to explain further the action mechanism from these experiments. The technique used here was growth experiments in shaking cultures with an experimental period of 4 days alternating with growth experiments in Warburg apparatus for shorter periods (6 hours). Especially the last-mentioned method was instructive for the investigation of the action mechanism of oxine. »Chemostat cells» were almost exclusively used.

In the earlier part of the investigation oxine base (Merck & Co.) was used, an alcoholic solution being added to the medium before sterilization. Later on, however, for practical reasons, the water soluble oxine sulphate (Eastman) was employed. In Warburg experiments only oxine sulphate has been used.

In turning from oxine to oxine sulphate a slight increase in fungistatic effect at the same molar concentration was noticed, probably owing to the fact that the oxine sulphate molecule contains two oxine molecules. In each of the results given below it is stated which substance was used in the particular experiments.

b) Oxine inhibition in shaking cultures

In the synthetic medium visible growth is obtained already after one day (Figure 3). An oxine concentration of $3 \cdot 10^{-5}$ M (as oxine base or oxine sulphate) is sufficient to prevent growth for an unlimited time. An oxine concentration of 10^{-5} M, on the other hand, gives a retardation in growth, visible growth appearing after 2—3 days as a rule, whereupon it continues as in a medium lacking oxine until limited by the glucose concentration. The duration of this lag, however, varies very greatly, from 1 to 4 days or more owing to hitherto unknown factors. The results of a typical experiment are shown in Figure 6.

From preliminary experiments for the purpose of obtaining information concerning the reason for the retardation of growth in 10^{-5} M oxine sulphate it seems probable that this behaviour is due to an adaptation to this concen-

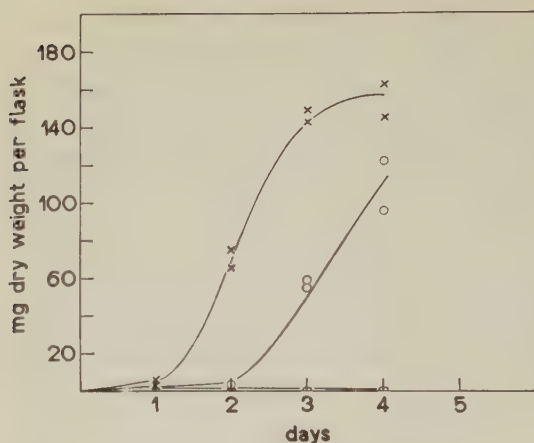


Figure 6. Growth of *C. albicans* in the synthetic medium with and without addition of oxine sulphate. \times — \times : control, \circ — \circ : 10^{-5} M oxine sulphate, \square — \square : $3 \cdot 10^{-5}$ M oxine sulphate.

tration of the drug rather than to the presence of resistant cells in the inoculation population. A gradual shortening of the lag phase was namely obtained by subcultivating cells from one 10^{-5} M culture to another (9 days—4 days—2 days). This, however, was also true, although to a lesser extent, with control cells without previous treatment with oxine. Further investigation on this subject is needed.

c) Oxine action in the presence of Cu^{2+}

The inhibition of growth obtained by addition of oxine derivatives is markedly strengthened in the presence of Cu. The results of an experiment in shaking cultures with varying amounts of oxine and Cu (added as copper sulphate) are given in Table 4. It is seen from the table that by the presence of approximately equivalent amounts of Cu and oxine total inhibition is obtained at an oxine concentration of about $5 \cdot 10^{-6}$ M. A tendency to a weaker action becomes apparent when Cu is added in excess. Cu itself is not inhibitory in these concentrations.

As this increased effect must be ascribed to the greater amount of highly stable Cu-oxine-complex, experiments were also performed with additions of a preparation of Cu-oxine-complex (furnished by AB. Pharmacia, Uppsala) to verify further the toxicity of this complex (Table 5).

On comparison between Tables 4 and 5 it is seen that the pure complex has somewhat greater effect than added oxine sulphate and copper sulphate. The results support the theory that the fungistatic action preferably must be due to the toxicity of the metal complexes in the solution and not to the oxine molecule itself or to trace element deficiency caused by complex formation in the medium.

Table 4. *The effect on growth of varying amounts of oxine and Cu in shaking cultures.*
 Dry weights in mg of duplicate flasks after 4 days on the shaking table.

<i>M</i> additions of oxine		<i>M</i> additions of Cu ²⁺					
		0	$3.2 \cdot 10^{-7}$	$1.6 \cdot 10^{-6}$	$7.9 \cdot 10^{-6}$	$1.6 \cdot 10^{-5}$	$7.9 \cdot 10^{-5}$
0	{	167	193	192	190	183	185
		177	184	187	176	193	190
10^{-6}	{	—	195	197	190	193	183
		—	195	196	172	185	178
$5 \cdot 10^{-6}$	{	—	185	125	0	0	17
		—	138	162	0	0	18
10^{-5}	{	178	144	0	0	0	0
		175	0	0	0	0	0

d) The fungistatic and fungicidal action of oxine

For the practical application of oxine inhibition it was of interest to know something about the degree of fungicity of the substance. For that purpose, cells which had been in contact with oxine in different concentrations in shaking cultures for various length of time were subcultivated to the glucose-agar medium. These cultures were placed in 37° C for at least two days, when the results were recorded. — As Cu increases the fungistatic action of oxine, the fungicidal action of simultaneous additions of Cu and oxine were also tested in some experiments.

The results of these experiments have been summarized in Table 6. It is seen from the table that oxine sulphate does not produce immediate and total fungicidal effect on *C. albicans* in the same concentrations which give fungistasis. Thus a concentration of $3 \cdot 10^{-5}$ *M* oxine or oxine sulphate, bringing about complete fungistasis, did not give total fungicity even in as long a contact time as 45 days. By increasing amounts of oxine the effect was somewhat increased. The addition of Cu somewhat enhances the fungicidal action of oxine (B: 20 days experimental period). A slightly increased effect

Table 5. *The action of added Cu-oxine-complex in comparison to that of oxine sulphate.*
 Experimental period: 4 days. 0 = no growth, (+) = slight growth in unweighable amount.
 The figures represent dry weights in mg.

Control	<i>M</i> additions of					
	oxine sulphate		Cu-oxine-complex			
	10^{-5}	$3 \cdot 10^{-5}$	$5 \cdot 10^{-7}$	10^{-6}	$5 \cdot 10^{-6}$	10^{-5}
170	(+)	0	168	(+)	0	0
169	(+)	0	165	(+)	0	0

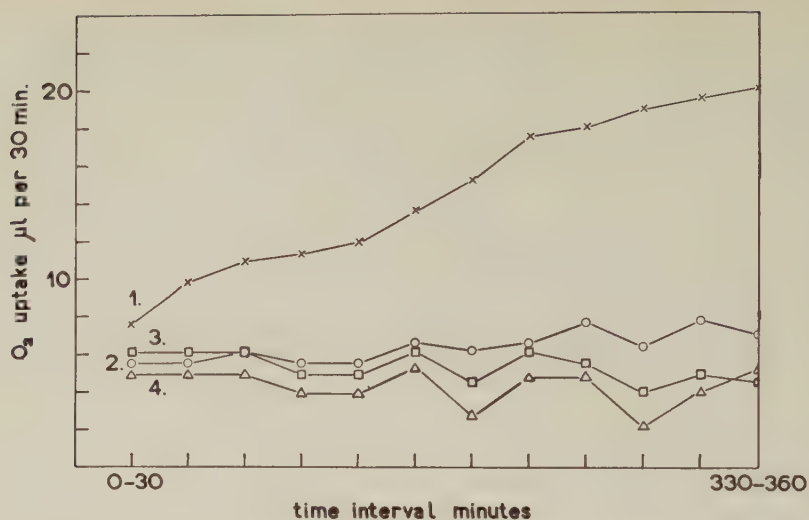
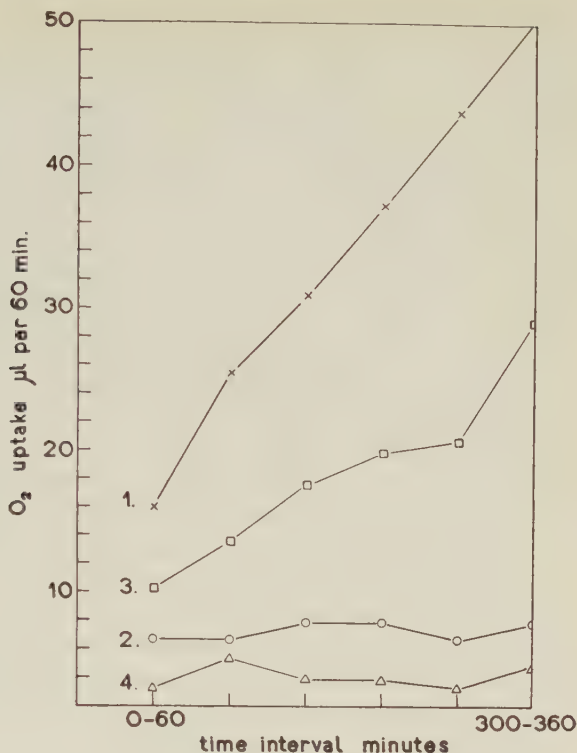


Figure 7. Oxygen uptake per unit of time (30 min.) in the basic medium and in glucose+phosphate with or without added oxine sulphate (10^{-5} M). — 1. X—X: Basic medium. 2. O—O: Basic medium+oxine sulphate. 3. □—□: Glucose+phosphate. 4. △—△: Glucose+phosphate+oxine sulphate.

Table 6. The fungicidal action of oxine sulphate (+ Cu). Growth on glucose-agar after subcultivating from shaking cultures containing oxine sulphate (and Cu) in varying amounts. A. Additions of oxine sulphate (and Cu) at the beginning of the experiment. B. Additions after one days growth. + = visible growth on glucose-agar after two days in 37° C. — = total absence of growth after two days in 37° C.

<i>M</i> additions of		Subcultivated after days						
oxine sulphate	Cu	4			45			
A.	$3 \cdot 10^{-5}$	—	+			+		
	$5 \cdot 10^{-6}$	$8 \cdot 10^{-5}$	+			+		
	10^{-4}	—	+			—		
	10^{-4}	$8 \cdot 10^{-5}$	—			—		
	10^{-3}	—	+			—		
	10^{-3}	$8 \cdot 10^{-5}$	+			—		
B.			5	10	15	20	25	30
	10^{-4}	—	+	+	+	+	+	+
	10^{-4}	$8 \cdot 10^{-5}$	+	+	+	+	—	—
	$5 \cdot 10^{-4}$	—	+	+	+	+	+	—
	$5 \cdot 10^{-4}$	$8 \cdot 10^{-5}$	+	+	+	—	—	—
	$7.5 \cdot 10^{-4}$	—	+	+	+	+	—	—
	$7.5 \cdot 10^{-4}$	$8 \cdot 10^{-5}$	+	+	+	—	—	—
	10^{-3}	—	+	+	+	+	—	—
	10^{-3}	$8 \cdot 10^{-5}$	+	+	+	—	—	—
			+	+	+	—	—	—

Figure 8. Oxygen uptake per unit of time (60 min.) by different amounts of cells in the basic medium with or without added oxine sulphate (10^{-5} M). — Cell densities: a and 2 a. 1. \times — \times : 2 a. 2. \circ — \circ : 2 a + oxine sulphate. 3. \square — \square : a. 4. \triangle — \triangle : a + oxine sulphate.



is noticed when Cu and oxine are added in equivalent amounts (e.g., A: 4 days experimental period).

e) Growth and inhibition in Warburg experiments

From the above-mentioned examples it is evident that oxine exerts an inhibiting action on the growth of *C. albicans*. For further study of oxine action it was of interest to know whether this growth inhibition was also accompanied by an inhibition of respiration.

A comparison of the oxygen uptake of a given amount of cells in glucose + phosphate and in the basic medium for growth experiments revealed that the O_2 uptake in the latter was much greater than in glucose + phosphate. An ever increasing amount of O_2 taken up per unit of time (30 min.) was noticed, while the O_2 uptake in glucose + phosphate was constant during the whole experimental period of 6 hours (Figure 7, curves 1 and 3). This behaviour seems to indicate cell multiplication in the basic medium during this relatively long experimental period. In a parallel experiment in which the same amount of cells were inoculated into flasks with the two substrates

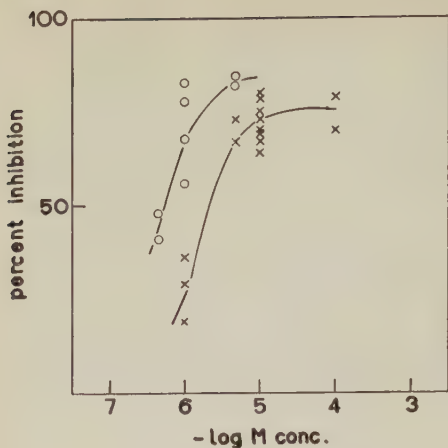


Figure 9. The dependence of the inhibition on the concentration of oxine sulphate and Cu-oxine-complex, respectively, at pH 5.5. —
 ×—×: oxine sulphate. o—o: Cu-oxine-complex.

and shaken on the shaking table for 6 hours at the same temperature this was also found to be the case. These cells were counted in a counting chamber at the beginning of the experiment and after 2, 4, and 6 hours. An increase to about three times the original number of cells was found in the basic medium, while only a slight increase was obtained in glucose + phosphate. As is seen from Figure 7 the O_2 uptake in the basic medium after 6 hours is about three times that in glucose + phosphate which is ascribed to the increased cell number during the experimental period.

If oxine sulphate in a concentration of $10^{-5} M$ was added an inhibition of the O_2 uptake of 60–70 per cent was obtained in the basic medium, while only 20 per cent inhibition was observed in glucose + phosphate. The O_2 uptake per unit of time in the basic medium furnished with oxine sulphate was kept at a constant level and coincides roughly with the curve for O_2 uptake in glucose + phosphate (see curve 2, Figure 7). This seems to indicate clearly that oxine in this concentration has a strong growth-inhibiting effect while respiration is not markedly influenced.

That a growth inhibition is exerted from $10^{-5} M$ oxine sulphate is further shown from Figure 8. Two different cell suspensions, the density of which was as 1 to 2, were added to the basic medium with or without oxine sulphate. It is evident from the figure that, firstly, the O_2 uptake is proportional to the cell density and, secondly, the O_2 uptake in the oxine experiments is kept constant at its original level indicating cell multiplication in the controls.

If the oxine inhibition obtained in these Warburg experiments is studied further it is found that at a pH of 5.5 a concentration of $10^{-5} M$ oxine sulphate gives maximal inhibition (about 70 per cent). The dependence of growth inhibition on oxine concentration is seen in Figure 9. It was shown that an increased concentration to $10^{-3} M$ did not cause increased inhibition.

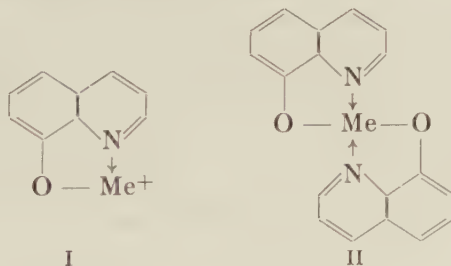
It is easily seen that this must be the case, as only growth and not respiration is inhibited in these concentrations. This is also in agreement with the observation that oxine action is fungistatic but not fungicidal in these concentrations and during this short time interval.

In shaking cultures total growth inhibition was not obtained with 10^{-5} *M* oxine but with $3 \cdot 10^{-5}$ *M*. The fact that 10^{-5} *M* oxine gives maximal inhibition in Warburg experiments depends on the fact that the Warburg experiment in its entirety falls in the lag phase of the corresponding shaking experiment where total inhibition still takes place.

In Warburg as in shaking experiments increased activity of oxine is obtained if Cu is added to the medium. Thus maximal inhibition is reached at a lower concentration (about 10^{-6} *M* Cu-oxine-complex). The inhibition is somewhat higher than that obtained with oxine sulphate (Figure 9). It may be possible that respiration inhibition here occurs owing to the greater toxicity of the Cu-oxine-complex.

f) Oxine action and pH

The following types of metal-oxine-complexes are formed with bivalent ions (e.g., Albert et al. 1953) and stand in equilibrium with each other, with the metal ions and with oxine in the solution:



As the complex formation occurs by substitution of a metal ion for a hydrogen ion, it is obvious that the pH of the medium markedly influences this formation and, as a consequence, the antimicrobial action of oxine. The stability of the complexes formed is further dependent on the metals concerned. Mellor and Maley (1947, 1948) have found the following order of decreasing stability of bivalent ions in metal complexes:



Further Irving and Williams (1948) determined the pH for 50 per cent precipitation of metal complexes. It can be concluded, according to these authors, that e.g., Cu may be precipitated at a pH of 2.5 while all other metals remain in the solution.

By studying oxine action at different pH values of the solution the use of

Table 7. *The influence of pH on oxygen uptake. μ l O₂ taken up in duplicate Warburg flasks after 6 hours.*

pH					
2.9	3.7	4.9	5.7	6.8	7.3
152.0	166.1	130.0	147.5	155.8	162.5
144.0	169.2	152.8	158.5	177.5	123.0

the synthetic medium in shaking cultures was not very instructive because of the large variations in pH during growth (Figure 3). In Warburg experiments, however, it was found possible to keep pH constant during the whole experimental period (6 hours). That the pH alone does not considerably influence the uptake of oxygen within the pH range 2.9 to 7.3 is shown in Table 7.

The inhibiting action of oxine, on the other hand, showed a very marked dependence on the pH within the same pH range. The percentage inhibition of oxygen uptake at different pH and different oxine concentration in various experiments is shown in Figure 10. It is clear that the effect is diminished by decreasing pH. Thus the inhibiting action of 10^{-6} M oxine sulphate at pH 5.5 is abolished already at pH 4.5. At a concentration of 10^{-5} M the inhibiting action begins to diminish at pH 4.7 and at pH 3.6 it has totally disappeared. At a concentration of 10^{-3} M oxine sulphate, on the other hand, the action is only slightly decreased by lowering pH and at pH 3.5 there is still about 70 per cent inhibition. The most probable explanation of this behaviour is that, in spite of the low pH, the toxic component is present in such amounts that inhibition occurs, that is, the equilibrium represented by the schematic formula



according to the law of mass action, is shifted to the right.

If the Cu complex is used principally the same behaviour is noticed (Figure 11). Owing to the greater stability of this complex inhibiting effect is still obtained at a pH about 3 and 10^{-5} M Cu complex.

g) Reversal of oxine action by metals

As mentioned earlier some metals have been reported to reverse oxine action. In experiments with *C. albicans* in shaking cultures the effect of additions of different metals have been investigated. The following concentration ranges of metals have been used:

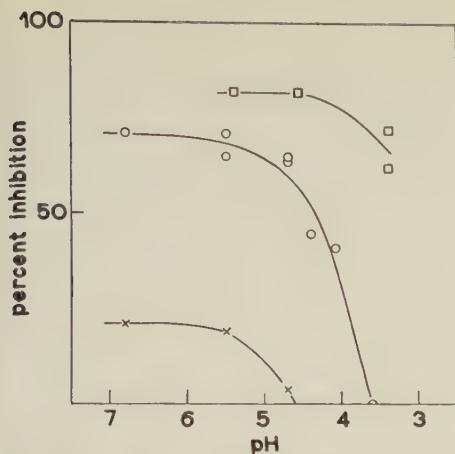


Figure 10. The inhibition by oxine sulphate as a function of pH at different additions of oxine sulphate. — \times — \times : 10^{-6} M, \circ — \circ : 10^{-5} M, \square — \square : 10^{-3} M oxine sulphate.

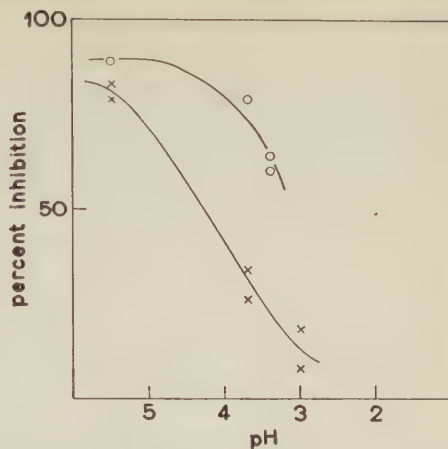


Figure 11. The inhibition by Cu-oxine-complex as a function of pH at different additions of Cu-oxine-complex. — \times — \times : 10^{-6} M, \circ — \circ : 10^{-5} M Cu-oxine-complex.

$$\text{Zn}^{2+} 2.8 \cdot 10^{-6} - 1.1 \cdot 10^{-4} \text{ M}$$

$$\text{Fe}^{3+} 3.6 \cdot 10^{-6} - 1.2 \cdot 10^{-4}$$

$$\text{Mn}^{2+} 3.6 \cdot 10^{-7} - 9.1 \cdot 10^{-5}$$

$$\text{Co}^{2+} 1.7 \cdot 10^{-6} - 3.2 \cdot 10^{-4}$$

$$\text{Ni}^{2+} 10^{-5} - 5 \cdot 10^{-3}$$

$$\text{Cu}^{2+} 3.2 \cdot 10^{-7} - 3.2 \cdot 10^{-4}$$

From these metals Zn, Fe, and Mn did not inhibit growth within the given concentration ranges, Co showed some inhibition at about $5 \cdot 10^{-5}$ M, while Ni was inhibitory only at $5 \cdot 10^{-4}$ M and Cu only in the highest concentration.

The concentration of oxine used in these experiments was just inhibiting growth. On addition of the above-mentioned metals, Fe, Zn, and Ni did not effect oxine inhibition, Cu strengthened this action, and Mn and Co showed the ability of reversing the oxine action. The results of an experiment with Mn and Co are shown in Table 8. Co and Mn were used in concentrations four times the concentrations of oxine sulphate (in one case only twice this concentration).

It is seen from the table that $3 \cdot 10^{-5}$ M oxine sulphate gives total inhibition, while initiating growth after 4 days is seen in $8 \cdot 10^{-6}$ and 10^{-5} M. The Mn additions do not effect growth, while Co gives increasing inhibition from the concentration of $3.2 \cdot 10^{-5}$ M. Mn as well as Co reverses oxine inhibition,

Table 8. *Reversal of oxine action by Co and Mn in shaking cultures. Dry weights in mg of at least duplicate flasks after 4 days cultivation.*

<i>M</i> additions of metal ions		<i>M</i> additions of oxine sulphate.			
Co ²⁺	Mn ²⁺	0	8 · 10 ⁻⁶	10 ⁻⁵	3 · 10 ⁻⁵
—	—	196	0 3	1 0	0 0
		185			
		193			
		194			
2 · 10 ⁻⁵	—	—	—	95 63	—
3.2 · 10 ⁻⁵	—	165 160	133 144	—	—
4 · 10 ⁻⁵	—	129 124	—	136 133	—
1.2 · 10 ⁻⁴	—	108 107	—	—	2 3
—	2 · 10 ⁻⁵	—	—	179 162	—
—	3.2 · 10 ⁻⁵	184 185	183 172	—	—
—	4 · 10 ⁻⁵	186 184	—	160 168	—
—	1.2 · 10 ⁻⁴	189 186	—	—	0 0

although the effect of Mn seems to be somewhat greater than that of Co. Thus the oxine inhibition is completely abolished in a Mn concentration twice the oxine concentration which is not the case with Co. In both cases it is obvious that the action of an oxine concentration definitely inhibiting growth ($3 \cdot 10^{-5}$ M) is not influenced to the same degree as a somewhat smaller concentration.

Reversal of oxine inhibition by Mn was also obtained although less pronounced when Cu-oxine-complex was used.

h) The application of oxine inhibition in medical therapy

The increasing number of cases of candidiasis in recent years has called forth a demand for substances preventing the overgrowth of *C. albicans*. In order to establish the universal effect of oxine on this fungus several other strains than the commonly used one (about 40 strains) isolated from human cases of candidiasis were tested in shaking cultures with the concentrations of oxine sulphate of 10^{-5} and $3 \cdot 10^{-5}$ M. No strain so far has resisted $3 \cdot 10^{-5}$ M oxine sulphate.

Repeated additions of serum proteins (10 per cent) did not markedly de-

crease the effect of oxine sulphate on the strain commonly used in these experiments.

The *in vivo* effect of oxine sulphate combined with copper sulphate in an ointment base (this preparation was produced by AB. Pharmacia, Uppsala) on *C. albicans* in vaginitis is now under investigation by S. Sjöstedt, M.D., Department of Obstetrics and Gynecology, Lund. The results of this work will be published elsewhere.

Discussion

As indicated in the preliminary experiments on growth factors mentioned above biotin was the only growth factor tested which showed an enhancing effect on the growth of the strain of *C. albicans* used. The trace element requirements are more complex in nature. It may be pointed out that although only Fe, Zn, and Mn have shown any effect on growth in these experiments it is not excluded that even other elements may be essential and furnished in sufficient amounts by glassware etc.

The growth and pH conditions obtained by culturing *C. albicans* in the synthetic medium are shown in Figure 3. As long as glucose was present and could be utilized by the fungus (i.e., until a dry weight of about 150 mg was reached, Figure 5) the pH fell rather rapidly, after that point increasing again to its original value. — Now it has been shown by Gale (1943), in studying the amino acid uptake by bacteria, that the amino acids were attacked either by decarboxylation or by deamination. A pH of 3–5 is favourable for the decarboxylase activity, while deaminase activity is obtained only at an alkaline pH. A decarboxylation is accompanied by a pH increase, a deamination by the opposite effect. — In the case reported here the increase in dry weight after the glucose has been used up seems to be due to utilization of asparagine. The pH in the medium favours decarboxylation. A pH increase, however, is obtained only after the acidifying effect of the glucose has disappeared, that is, after 3–4 days when the glucose has been utilized. A further alkalization above pH 5.5 is prevented by the fact that the decarboxylase activity as well as the deaminase activity decrease in this range of pH and balance each other.

If the same way of reasoning is applied to the experiment with the buffered medium (Figure 4) it seems evident that the decarboxylase activity as well as the deaminase activity are kept very low during the whole experimental period, owing to the pH conditions. The lower dry weights obtained here, therefore, seem to be due to the less complete utilization of asparagine.

As mentioned in the introduction the search for an inhibiting agent was one of the most important purposes of this investigation. Table 3 summarizes

briefly the results of these more or less random investigations which were carried out throughout the course of this study. — Desthiobiotin, an anti-metabolite for certain biotin-requiring organisms (e.g., Goldberg et al. 1947), was shown to be utilized to the same degree as biotin itself by *C. albicans*. — Several auxins and related compounds with very pronounced effects on higher plants showed very weak inhibiting action on *C. albicans*, which may be ascribed to the lack of an auxin mechanism similar to that in higher plants. The same may be the cause for the slight effect of coumarin derivatives. — On the other hand, the effect of the quaternary ammonium compounds was rather marked. It is known, however, that a certain irritating effect on the skin accompanies the action of these substances. Beside that the activity is often diminished in the presence of serum proteins. — The inhibiting action of selenium compounds was not studied further although interesting theoretical problems concerning competition between Se and S are offered here (see further e.g., Fels and Cheldelin 1949, Postgate 1949, 1952, Shrift 1954).

The oxine inhibition — the most important inhibiting agent in this study — was found to be almost entirely fungistatic (Table 6). Albert et al. (1953), however, found that oxine showed rapid bactericidal action in the same concentration which brought about bacteriostasis. On the other hand, Mason (1948) on investigating the oxine inhibition of germinating spores of *Stemphylium sarcinaeforme* has reported that oxine sulphate killed the spores only in concentrations $> 2.5 \cdot 10^{-4}$ M. In the case of *C. albicans* the results agree more with those obtained by Mason than with those of Albert et al. which may be due to the different organism material.

The fungistatic activity of oxine also seems to agree with the fact that growth inhibition but not respiration inhibition was obtained at least when moderate amounts of oxine were used. This experience is further supported by the results obtained by other authors on different organisms. Thus Klöpping (1951) obtained respiration inhibition on *Aspergillus niger* and *Penicillium italicum* only by oxine concentrations $> 3 \cdot 10^{-3}$ M. The same was found by Gottlieb and Davis (1948) in studying growth of *Sclerotinia fructicola* and by Gale (1949, 1951) who investigated glutamic acid assimilation in *Staphylococcus aureus*.

As to the mode of action of oxine, the theory of trace element deficiency alone does not seem to be valid, since the same effect was obtained by adding pure metal complexes (Mason 1948, Sexton 1949, etc.). This is supported by the present investigation in which Cu-oxine-complex was used whereby an increased inhibition capacity was established. Owing to the greater stability of the Cu complex other metals are expected to be free in the solution and available for the organism. Furthermore, when the pH was lowered to 3.5 the fungus was still inhibited by Cu-oxine (Figure 11), while the inhibition ob-

tained by the same concentration of added oxine sulphate was abolished at this pH (Figure 10). This behaviour indicates clearly not only the greater stability of the Cu complex but also the fact that the complex itself is primarily responsible for the toxic action of oxine. The oxine molecule itself seems to be nontoxic as indicated by Rubbo et al. (1950). A decreased toxicity was noticed also in these experiments when oxine was added in excess in relation to Cu which indicates a lower toxicity of the oxine molecule itself than of the oxine complexes, provided that both molecules are taken up by the cell at the same rate.

The investigations made by Albert et al. (1953, 1954) seem to prove that this toxic effect of oxine complexes is exerted *within* the cell. These authors showed that only chelating properties parallel to a relatively high partition coefficient between oil and water (lipophilic character) assured antibacterial action. According to Albert et al. the saturated oxine complex (II, p. 707) is lipid-soluble but nontoxic, while the unsaturated complex (I) is lipid-insoluble and toxic. In order to be able to exert its toxic action this latter complex must, however, enter the cell. This is accomplished by the entrance of the saturated complex into the cell where it, according to the law of mass action, gives rise to a certain amount of the toxic component if suitable conditions between metal and oxine are present there. The unsaturated and charged complex then exerts its action by oxidizing a -SH-system, which reaction is catalyzed by the presence of Fe- and Cu-complexes, thus disturbing the cell division mechanism.

The results obtained here are in agreement with those of Albert et al. in so far as a sulphonic acid derivative of oxine does not inhibit growth, indicating a lowering of the lipophilic character of the molecule (Table 3). Furthermore, excess of Cu in relation to oxine as well as excess of oxine in relation to Cu shows less inhibition than if equivalent amounts were used. (Tables 4 and 6). This seems to indicate a decrease of the toxic component in the cell in favour of the relatively nontoxic one. The possibility that also the saturated complex exerts any toxic action, however, does not seem to be quite excluded. A hypothesis of Erlenmeyer et al. (1953) concerning the action of complex-Cu as distinguished from that of free Cu ions here furnishes a plausible explanation of some of the effects found. Erlenmeyer's hypothesis aims at an alteration of the ionic conditions in such a direction that a competitive antagonism between Cu in a complex and an enzyme metal was possible. This hypothesis was further supported by the results of Vajda and N6grádi (1954), showing a competitive antagonism between enzyme Co and complex Cu.

As suggested by Albert et al. oxine interferes with a -SH-system of the cell. The cell division mechanism in yeast, according to Nickerson and van

Rij (1949), is regulated by the amount of -SH-groups. A maintenance of a high proportion of -SH-groups promotes cell division. The addition of Co in subinhibiting concentrations influences this mechanism in favour of mycelial production, that is, the equilibrium $\text{-SH} \rightleftharpoons \text{-S-S-}$ is shifted to the right. Such a Co effect on the cell division mechanism seems to be valid for yeasts as well as for bacteria.

The general tendency of Co to counteract oxine action as reported in literature seems to be connected with this cell division mechanism. (The specificity of Co action is here striking: Ni, having similar chemical and stability conditions, did not show this reversing effect on oxine action.) In this connection, it is characteristic that Co is reported to counteract oxine action in the cases where bacteria are used as experimental material as well as in this investigation on the yeast phase of *C. albicans*, while a similar Co effect was not obtained for *Aspergillus niger* (Anderson and Swaby, 1951), where a cell division mechanism of the kind present in bacteria and yeast is lacking.

Beside Co, Mn has been shown here to reverse oxine action. This Mn action seems to be even more pronounced than the corresponding Co effect. Mn effect of this kind has earlier been reported only by Gale (1949). Even Co had this effect, although to a lesser degree, as well as Fe and Mg. According to Gale the connection between oxine and the bacterial receptor must be of the same strength as the forces in the Mg complex, as even Mg is able to bring about a neutralizing effect. As Mn, the complex stability of which is closely related to that of Mg, had greater effect than other metals it seemed possible that it had some function in the glutamic acid assimilation. This was also shown to be the case, at least in growing cells. The ability of Mn to counteract oxine action, therefore, seems to be due to its co-operation in the amino acid assimilation.

In the medium used here asparagine was added as a source of nitrogen. It is not excluded that a parallel to Gale's Mn action in glutamic acid assimilation is presented here. While Co antagonism may be explained on stability reasons, this explanation for Mn action is not very probable, because the stability of Mn-oxine is much less than that of Co-oxine, although its antagonistic effect is greater than that of Co. This theory is further supported by the fact that a certain counteraction of Cu-oxine is obtained by Mn.

From the discussion presented above it thus seems possible that Co and Mn action on oxine inhibition acts on different processes. The possibility of Co and Mn compensating for each other in a certain enzyme system, however, may not be overlooked (cf. Starkey, 1955).

Further research on oxine inhibition and its counteraction by metals on the yeast phase as well as on the mycelial phase of *C. albicans* is needed to give more information about the problems concerned here.

Summary

The growth of the yeast phase of *C. albicans* in a synthetic glucose-asparagine medium has been studied. For the purpose of obtaining a homogeneous inoculation material the fungus was cultured in a so-called chemostat of the type described by Novick and Szilard (1950). Growth and inhibition experiments inoculated with the homogeneous cells thus obtained were than performed in shaking cultures as well as in Warburg experiments.

Growth and pH conditions in the synthetic medium are discussed in relation to the components of the medium.

The inhibiting action of about 35 different substances, most of which showed negligible activity, is presented. The action of 8-hydroxyquinoline (oxine), which was more pronounced, was studied more in detail.

Oxine totally inhibits growth in shaking cultures in a concentration of $3 \cdot 10^{-5}$ M. The increased inhibition by addition of Cu earlier established was confirmed, especially by studying the effect of pure Cu-oxine-complex parallel to that of oxine.

Oxine action (as well as Cu-oxine action) is found to be fungistatic rather than fungicidal in moderate concentrations in these experiments.

As oxine action is highly dependent on the pH, showing diminished effect with decreasing pH, the conditions in the Warburg experiments, where the pH was kept constant during the experimental period of 6 hours, permitted a more careful study of the inhibiting action of oxine on growth and its relation to pH than was possible in shaking cultures. The action was found to be almost entirely growth inhibition, respiration not being affected at moderate concentrations. This behaviour was found to agree with its fungistatic action.

Oxine action was found in these experiments to be counteracted by additions of Co and Mn. An attempt to explain this behaviour is presented in relation to results earlier obtained in this field.

The application of oxine action in vivo in order to prevent overgrowth of *C. albicans* in vaginitis is under investigation. The results seem to be promising.

The author is indebted to Professor H. Burström at this institute and to Dr. G. Fåhræus, Institute of Microbiology, Royal Agricultural College, Uppsala, for valuable advice and encouraging discussions. Sincere thanks are also due to Å. Nordén, M.D., Medical Clinic, Lund, for suggesting the problem and providing the organism, and to Dr. N. Fries, Institute of Physiological Botany, Uppsala, for valuable help and suggestions for the use of the chemostat.

References

- Albert, A., Rubbo, S. D., Goldacre, R. J. & Balfour, B. G.: The influence of chemical constitution on antibacterial activity. Part III: A study of 8-hydroxyquinoline and related compounds. — *Brit. J. Exptl. Path.* 28: 69. 1947.
- , Gibson, M. I. & Rubbo, S. D.: The influence of chemical constitution on antibacterial activity. Part VI: The bactericidal action of 8-hydroxyquinoline (oxine). — *Brit. J. Exptl. Path.* 34: 119. 1953.
- , Hampton, A., Selbie, F. R. & Simon, R. D.: The influence of chemical constitution on antibacterial activity. Part VII: The site of action of 8-hydroxyquinoline (oxine). — *Brit. J. Exptl. Path.* 35: 75. 1954.
- Anderson, B. I. & Swaby, R. J.: Factors influencing the fungistatic action of 8-hydroxyquinoline (oxine) and its metal complexes. — *Austr. J. Sci. Res. B* 4: 275. 1951.
- Burkholder, P. R.: Vitamin deficiencies in yeasts. — *Am. J. Bot.* 30: 206. 1943.
- Erlenmeyer, H., Bäumlér, J. & Roth, W.: Metallkomplexe und tuberkulostatische Aktivität. — *Helv. Chim. Acta* 36: 941. 1953.
- Fels, G. & Cheldelin, V. H.: Selenate inhibition studies. III. The role of sulfate in selenate toxicity of yeast. — *Arch. Biochem.* 22: 402. 1949.
- Gale, E. F.: Factors influencing the enzymic activity of bacteria. — *Bact. Revs.* 7: 139. 1943.
- The assimilation of amino acids by bacteria. 8. Trace metals in glutamic acid assimilation and their inactivation by 8-hydroxyquinoline. — *J. Gen. Microbiol.* 3: 369. 1949.
- & Paine, T. F.: The assimilation of amino acids by bacteria. 12. The action of inhibitors and antibiotics on accumulation of free glutamic acid and the formation of combined glutamate in *Staphylococcus aureus*. — *Biochem. J.* 48: 298. 1951.
- Goldberg, M. W., Sternbach, L. H., Kaiser, S., Heineman, S. D., Scheiner, J. & Rubin, S. H.: Antibiotin effect of some biotin homologs. — *Arch. Biochem.* 14: 480. 1947.
- Gottlieb, D. & Davis, D.: Investigation on the mechanism of fungicidal activity. — *Phytopath.* 38: 10. 1948.
- von Hofsten, B., von Hofsten, A. & Fries, N.: Continuous liquid culture of the fungus *Ophiostoma multiannulatum*. — *Exp. Cell Res.* 5: 530. 1953.
- Irving, H. & Williams, R. J. P.: Order of stability of metal complexes. — *Nature* 162: 746. 1948.
- Kligman, A. M.: Are fungus infections increasing as a result of antibiotic therapy? — *J. Am. Med. Assn.* 149: 979. 1952.
- Klöpping, H. L.: Chemical constitution and antifungal action of sulphur compounds. Utrecht. 1951.
- Lilly, V. G. & Leonian, L. H.: The interrelationship of iron and certain accessory factors in the growth of *Rhizobium trifolii*, strain 205. — *J. Bact.* 50: 383. 1945.
- Mason, C. L.: A study of the fungicidal action of 8-quinolinol and some of its derivatives. — *Phytopath.* 38: 740. 1948.
- Mellor, D. P. & Maley, L.: Stability constants of internal complexes. — *Nature* 159: 370. 1947.
- , — Order of stability of metal complexes. — *Nature* 161: 436. 1948.
- Monod, J.: La technique de culture continuée. Théorie et application. — *Ann. Past.* 79: 390. 1950.
- Nickerson, W. J. & van Rij, N. J. W.: The effect of sulphhydryl compounds, penicillin, and cobalt on the cell division mechanism of yeasts. — *Biochim. et Biophys. Acta* 3: 461. 1949.

- & Mankowski, Z.: Role of nutrition in the maintenance of the yeast shape in *Candida*. — *Am. J. Bot.* 40: 584. 1953.
- Novick, A. & Szilard, L.: Description of the chemostat. — *Science* 112: 715. 1950.
- — Experiments with the chemostat on spontaneous mutations of bacteria. — *Proc. Natl. Acad. Sci.* 36: 708. 1950 a.
- Philipson, T.: Über die quantitative Bestimmung reduzierender Zucker. — *Arkiv för kemi, mineralogi och geologi.* 16 A: 22. 1943.
- Postgate, J. R.: Competitive inhibition of sulphate reduction by selenate. — *Nature* 164: 670. 1949.
- Competitive and non-competitive inhibitors of bacterial sulphate reduction. — *J. Gen. Microbiol.* 6: 128. 1952.
- Rubbo, S. D., Albert, A. & Gibson, M. I.: The influence of chemical constitution on antibacterial activity. Part V: The antibacterial action of 8-hydroxyquinoline (oxine). — *Brit. J. Exptl. Path.* 31: 425. 1950.
- Schuler, W. & Meyer, R.: Der Kobalt-Antagonismus der antibakteriellen 8-Oxy-chinolin-Wirkung in Atmungsversuchen. — *Schweiz. Zeitschr. Path. Bakt.* 13: 463. 1950.
- Sexton, W. A.: Chemical constitution and biological activity. London, 1949.
- Shrift, A.: Sulfur-selenium antagonism. I. Antimetabolite action of selenate on the growth of *Chlorella vulgaris*. — *Am. J. Bot.* 41: 223. 1954.
- Sorkin, E., Roth, W., Kocher, V. & Erlenmeyer, H.: Über die Wirkung von Metallionen auf die tuberkulostatische Aktivität des Oxins und der PAS. — *Experientia* 7: 257. 1951.
- Starkey, R. L.: Relations of micronutrients to development of microorganisms. — *Soil Sci.* 79: 1. 1955.
- Vajda, M. & Nógrádi, T.: Komplex-Stabilität und tuberkulostatische Aktivität einiger 8-Oxychinolin-Derivate. — *Experientia* 10: 373. 1954.
- Waring, W. S. & Werkman, C. H.: Growth of bacteria in an iron-free medium. — *Arch. Biochem.* 1: 303. 1943.
- Zentmeyer, G.: Mechanism of action of 8-hydroxyquinoline. — *Phytopath.* 33: 1121. 1943.
- Inhibition of metal catalysis as a fungistatic mechanism. — *Science* 100: 294. 1944.

The Effect of Some Dithiocarbamyl Compounds on the Metabolism of Fungi

By

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Contents

Preface	720
I. The Chemistry of the Dithiocarbamyl Compounds	722
II. Earlier Investigations Regarding the Physiological Effects of the Dithiocarbamyl Compounds	724
III. Analytical Methods for the Dithiocarbamyl Compounds	734
IV. Studies on the Stability of the Dithiocarbamyl Compounds, and their Behaviour in Biological Systems	737
A. Reactions in a Mineral Salts Nutrient System	737
B. Reactions when in Combination with Cellular Extracts	751
C. Absorption Spectra of Dithiocarbamyl Compounds in Yeast Suspensions	754
V. Attempts to determine the Uptake of Dithiocarbamyl Compounds by Yeast Suspensions	755
VI. The Effect of Dithiocarbamyl Compounds on the Oxidative Reactions in Intact and Resting Yeast Cells	760
A. Methods	760
B. Introductory Experiments with Glucose as the C-source	761
C. The Oxidation of Acetate. The Effect of Heavy Metals as Synergists for Na-DMDT	763
D. The Inversion Phenomenon	767
E. The Effect of Other Dithiocarbamyl Compounds on the Acetate Oxidation ..	771
F. The Effect of Histidine and Chelating Agents on the Na-DMDT Action	773
G. The Effect of Na-DMDT on Various Oxidative Processes in Intact Cells, in Comparison with the Effect on Acetate Oxidation	775
VII. The Effect of the Dithiocarbamyl Compounds on the Pasteur Mechanism in Intact Cells	783
VIII. The Effect of the Dithiocarbamyl Compounds on the Anabolic Processes	786
A. Methods	785

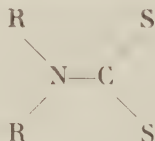
B. The Effect of Na-DMDT on Growth, Respiration of Growing Cells, Glucose Consumption and Acetate Fixation	787
C. Nitrogen Assimilation	794
IX. Some Observations Regarding the Effect of Dithiocarbamyl Compounds on Enzyme Reactions	796
A. Introduction	796
B. Succinic Oxidase	796
C. Alcohol Dehydrogenase (ADH)	798
D. The Acetate-Activating System	799
E. Preliminary Investigations Regarding the Formation of Hydrogen Sulfide from Dithiocarbamate in the Presence of Hydroxylamine and Yeast	801
X. Discussion	805
A. The Chemical Reactions	805
B. Experiments with Respiratory Systems	807
C. The Experiments with Growing Cells	810
D. Biochemical Considerations	814
E. Fungicidal Activity and Chemical Structure	819
F. Conclusions and Generalizations	822
Summary	824
References	827

Preface

Since 1927, when Tisdale and Williams patented the use of dithiocarbamates as fungi- and insecticides, this group of organic compounds has played a very important role in the fight against plant diseases in agriculture, as well as against harmful fungi in industry. A large number of different derivatives are sold under various trade names, and new types are still frequently patented.

The compounds are characterized by their high toxicity towards fungi even in minute concentrations, while most of them are comparatively harmless to plants (Goldsworthy, Green and Smith, 1943) and warm-blooded animals (Brieger and Hodes, 1949; Lehman, 1950). The manner in which they exert their fungicidal effect has, however, been virtually unknown, although several theories have been put forward.

Due to the large number of dithiocarbamyl derivatives described, it has been found necessary to concentrate the present investigations on one of the main types, viz. the secondary monoaminoderivatives, with the characteristic group:



Of these, the dimethylamine derivatives have been most thoroughly studied, while the unsubstituted dithiocarbamate, and the diethyl and dipropyl deri-

vatives have been brought in for comparison when it has been of interest. The types of derivatives which have been studied are the sodium salt, the zinc and cupric complexes of the dithiocarbamic acids, the thiuram disulfides, and the thiuram monosulfides. For these compounds, the following abbreviation system has been worked out, partly in agreement with earlier literature (e.g. Horsfall, 1945):

Na-DT:	Sodium dithiocarbamate
Na-DMDT:	Sodium dimethyldithiocarbamate
Zn-DEDT:	Zinc diethyldithiocarbamate
Cu-DPDT:	Cupric dipropyldithiocarbamate
TMTD:	Tetramethyl thiuramdisulfide
TMTM:	Tetramethyl thiurammonosulfide
etc.	

Otherwise, abbreviations common in biochemical literature have been used.

Test Organism. In investigations emerging from a practical problem, it is natural and also correct to use an organism which is of practical importance — in this case, an organism which is harmful and which has been successfully attacked by the group of fungicides to be studied. A number of organisms have been used in earlier work, both plant parasites and harmful saprophytes. In the present work, it was first intended to use *Aspergillus niger* as the test organism. However, acid-producing organisms were soon found not to be convenient in these investigations, due to the instability of dithiocarbamates in acid solution. If *Aspergillus niger* were to be used regardless, the major problem in the whole investigation probably would have been to make a perfect buffering system for the culture solutions, not allowing pH to fall below 6. As this would seem somewhat futile, the possibility of selecting one or more other test organisms was considered. Of the various fungi (their practical importance left aside), the *yeasts* could be considered ideal as test organisms, for a number of reasons. They are moderate or low acid producers, and the growth can be followed easily by photometric methods, or by counting. A yeast suspension is also from a physiological point of view much more satisfactory to work with than a mycelial mat, or submerged pellets of mycelium. *Saccharomyces cerevisiae* (baker's yeast) was thus selected as the test organism. By purchasing it in the fresh state, the problem of maintaining a sufficient quantity of physiologically well defined test material could easily be solved. As perhaps the most important factor, the large amount of knowledge regarding the physiology and biochemistry of baker's yeast must be considered. The cytological knowledge is more scanty.

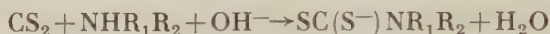
As a member of the large and heterogeneous group of organisms making up the fungi, it is probably as representative as any other single member.

The baker's yeast used was purchased from A/S De Norske Gjør- og Spritfabrikker, Oslo. By microscopic examination we found that it contained a small amount of bacteria and of *Oospora lactis*, probably negligible for the investigations carried out in this work.

During the period of investigation when yeast was used (April 1954—March 1955) the same strain of yeast (from a single cell isolate) was used by the factory for propagation and production of yeast.

I. The Chemistry of the Dithiocarbamyl Compounds

The dithiocarbamates are derived from the carbamates by replacing the two oxygen atoms with sulfur. They are easily prepared by adding ammonia or an amine to carbon disulfide in alcohol (Delépine, 1908):

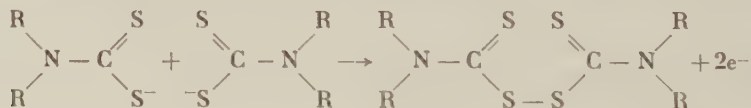


The free dithiocarbamic acids are unstable. The alkali and earth alkali salts are easily soluble in water, with alkaline reaction. Na-DMDT crystallizes well, with 3 moles of water. The crystal water is partly lost by standing or heating, and the compound then also undergoes partial decomposition.

The dissociation constant for H-DEDT is by Gregg and Tyler (1950) calculated to be $2.9 \cdot 10^{-6}$ in 60 per cent ethanol at 25°C (from polarographic data).

The heavy metal compounds of the dithiocarbamic acids are coloured (except for the Zn compounds), and sparingly soluble in water, but more soluble in nonpolar solvents like chloroform and benzene.

The dithiocarbamates are easily oxidized to the corresponding thiuram disulfides (bis[thiocarbamyl]disulfides) (Braun, 1902), i.e., by alcoholic iodine, persulfate or tetrathionate:



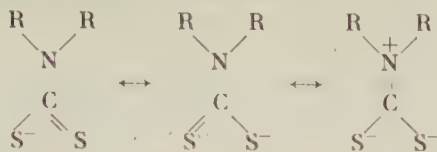
The thiuram disulfides are sparingly soluble in water and alcohol, but easily soluble in nonpolar solvents. They crystallize well, and are fairly stable. TMTD melts with some decomposition at 146°C ; it is practically colourless.

By treatment of the thiuram disulfides with an excess of KCN in warm alcohol, one atom of sulfur is split off, and the corresponding thiuram monosulfide is formed (Braun and Stechele, 1903):

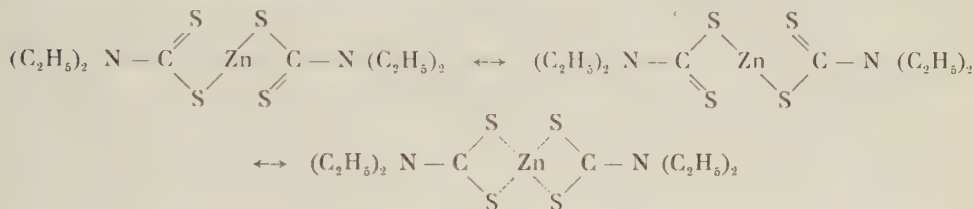


TMTM is yellow, more easily soluble than TMTD, crystallizes well and melts at 106°C .

Structurally, all these compounds are interesting. The dithiocarbamate ion must be supposed to resonate between the structures:

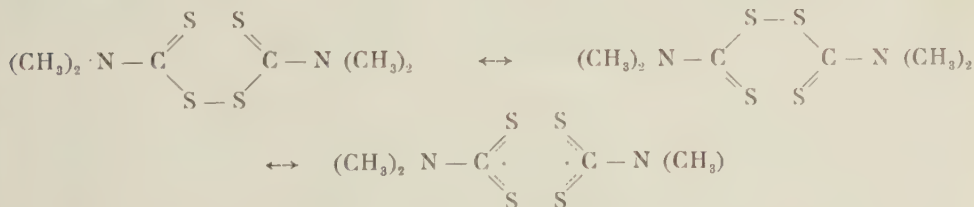


This is probably the reason why the compound is far more stable as ion than as free acid, where the resonance possibilities are considerably reduced. Many structures have been proposed for the heavy metal complexes. Koch (1949) has studied the absorption spectra of the compounds. He stated that the UV-spectrum of Zn-DEDT was characteristically different from that of Na-DEDT. While Na-DEDT is a typically ionized compound, Zn-DEDT contains covalent bondings, and he supposed a hybridisation between two symmetrical structures:



This is in accordance with modern conceptions, according to which these complexes are metal chelates, with the dithiocarbamyl groups as bidentate, resonating ligands, the resonance being between the two sulfur atoms. Both Cu(II) and Zn have normally the coordination number of four, but the Cu chelates are of the square type, while the Zn chelates are tetrahedral. As there should be no steric hindrances or other objections against a normal configuration in this case (the valence angle for sulfur may be a bit abnormal), it can be assumed that, for example, in Cu-DMDT the sulfur atoms are lying in a square, while in Zn-DMDT they make out the corners of a tetrahedron (cf. Martell and Calvin, 1952).

Koch (1949) also discussed the structure of TMTD by means of the absorption spectrum. It had no similarity with the spectrum of methyl dimethyldithiocarbamate, which could be believed to contain the »half-chromophor». The reason for this must lie in particular resonance conditions within the molecule. He gave the following possibility:



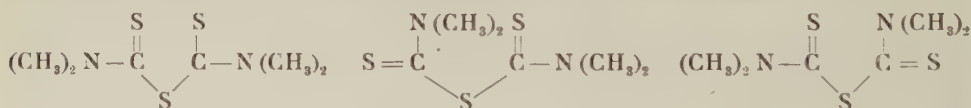
There can be no doubt regarding the normal, straight-chained nature of the disulfide bridge in TMTD. This is in accordance with the method of synthesis (oxidative coupling of two SH-groups). However, a dissociation of the molecule in free

radicals could be possible. These radicals could be well stabilized by resonance. The idea of dissociation is in agreement with the opinion of Bergem (1948).

Craig et al (1950), (1951) have, however, found that the molecular weight of TMTD in benzene at infinite dilution is 235, and in 5.4 per cent solution 257 ± 2 (theoretically, the molecular weight is 240.41). This, they state, should rather point in favour of association. They also prepared labeled TMTD by means of TMTM and isotope sulfur, and found that all sulfur atoms in TMTD are equivalent. The probable structure proposed by them was a hybridization between the same ground structures as given by Koch, and resulting in a planar ring formation.

On being heated, TMTD forms carbon disulfide, elementary sulfur and tetramethyl thiourea. This rather peculiar reaction was explained by Koch (1949) as being due to a transfer of a single electronpair from one dimethyl dithiocarbamyl group to the other in a cyclic process. One has then to assume that the ring with four sulfur atoms is broken, and that the molecule can rotate round the C—S bonds, so that the nitrogen atom from one dithiocarbamyl group can come close to the sulfur atoms from the other. Koch stated, however, that this explanation cannot account for the surprisingly featureless spectrum, which only contains an inflection point around 2800 Å.

TMTM was also studied by Koch. This compound has a strong and broad absorption band around 2700 Å, stretching into the visible region. By careful consideration of the spectrum, he concluded that in TMTM there must be a coplanar configuration of the two $=NC=S$ groups, with an energy barrier prohibiting free rotation around the central sulfur atom. Three stereoisomers could then be expected:



However, only one stable modification of TMTM is known. It would perhaps not be far-fetched to believe that this is a resonance hybrid with a planar five-membered ring.

Yamada and Mizuno (1941 a and b) have determined the crystal structure of TMTD and TMTM by x-ray analysis. Both belong to the monocline holohedral class C_{2h}^{-2}/m . Both have four molecules per single cell. The symmetry conditions indicated a valence angle of the S atoms different from 180° .

II. Earlier Investigations Regarding the Physiological Effects of the Dithiocarbamyl Compounds

As already mentioned, the discovery of the fungicidal effect of the dithiocarbamates was made in 1927. Since then, a vast amount of work has been published regarding biocidal studies and practical applications of the various members of this group of compounds. In this review, papers dealing with the practical problem of controlling harmful fungi in agriculture or industry have been omitted, insofar as they do not report observations from which conclusions of physiological interest may be drawn. On the other hand, the

effects of the dithiocarbamyl compounds on other organisms than fungi have been reviewed, as it is felt that from the findings, e.g. of the effects of Antabuse on animal enzyme systems, important general conclusions may be drawn.

Zn-DMDT and TMTD had for some time been used at vulcanisation accelerators when their fungicidal and insecticidal value was discovered, and early workers compared the effect of various compounds as vulcanisation accelerators and as fungicides (Marsh, 1938, Dimond and Horsfall, 1943). General reviews about dithiocarbamates as fungicides have been written by McCallan (1946), Tisdale and Flenner (1951), and recently by van der Kerk (1953).

Tisdale and Flenner (1942) studied the effect of several thiuram sulfides and dithiocarbamates on *Ustilago hordei*, *Fomes annosus*, *Aspergillus niger* and *Trichophyton* sp. A wide range of toxicity of the different compounds was found; generally, the more soluble compounds were the more toxic. Tisdale and Flenner studied the insecticidal effect of TMTD against the Japanese beetle, and observed that it was due to a paralysis of the forelegs and mouth parts of the insects.

A hypothesis to explain the effect of *dithiocarbamates* was put forward by Parker Rhodes (1943), who suggested that the effect might be due to the splitting of the compounds into simpler substances, such as carbon disulfide and secondary amines. This hypothesis is not satisfactory, as all decomposition products are considerably less toxic than the dithiocarbamyl compounds themselves (McCallan, Miller and Weed, 1952, Palmer, Greenlee and Baldwin, 1952).

In recent years, it has been believed that the fungicidal effect must be connected with the ability to form heavy metal complexes. Thus, the toxic action should primarily be due to a deprival of copper ions from the fungus. In accordance with this idea, originating from Horsfall (1945), van Raalte (1952) found that the fungistatic effect of Na-DMDT on *Fusarium coeruleum* and *Aspergillus niger* was counteracted by cupric ions, and that the antagonistic effect had its maximum when the amount of copper sulfate added corresponded stoichiometrically to the amount of Na-DMDT. Lees (1948) found that Na-DEDT reduced nitrification in soil, and he explained this as being due to a binding of the copper in the soil, rendering it deficient in that metal. The inhibition was reversed by addition of Cu, and partly by Mn salts. Other investigators have, however, found that even Cu-DMDT is toxic in some instances, (Goldsworthy, Green and Smith, 1943).

Liebermeister (1950 a and b) studied the effect of sulfur-containing chemotherapeutical compounds on bacteria, and stated that the effect of copper-binding compounds is not due to the removal of copper, but to the establish-

ment of copper complexes. Schrauffstätter (1950) was not able to find a general and definite connection between complexing ability and antibacterial effect. Hagelloch and Liebermeister (1951) found that the majority of a large number of compounds with the group :NC(:S)- with complex-forming abilities, had antibacterial effect. Sorkin, Roth and Erlenmeyer (1952) found that addition of copper to tuberculostatic compounds increased their toxicity towards bacteria up to ten times in vitro, due to formation of copper complexes. Also, Jeney et al (1954) stated that there is a distinct relation between chelate forming ability and bacteriostatic effect.

Enzymatic investigations have shown that Na-DEDT inhibits copper-containing enzymes like catechol oxidase (tyrosinase), laccase and ascorbic acid dehydrogenase (Kubowitz, 1937, Keilin and Mann, 1939, Stotz, Harrer and King, 1937, Rothchild and MacVicar, 1949). It is supposed that the inhibition is due to the removal of copper from the prosthetic group of the enzyme.

Na-DEDT is also stated to inhibit alkaline phosphatase (Roche, 1950). This enzyme is activated by magnesium and other bivalent cations, e.g. zinc, and it is also inhibited by other chelating agents like Versene (ethylene diamine tetraacetic acid) (Freimann, 1953).

The dithiocarbamates inhibit only weakly cytochrome oxidase (James and Garton, 1952).

Klöpping (1951) has studied the effect of Na-DMDT, Na-DEDT and a number of other compounds on the oxygen uptake by mycelia of *Aspergillus niger* and *Penicillium italicum*. He found an effect on oxygen consumption only in concentrations which far exceeded those that completely inhibited the growth. This is the fact even though mycelium is more resistant towards the dithiocarbamyl compounds than the spores (Sijpesteijn and van der Kerk, 1952), with which the comparative growth experiments were made.

Weed et al (1953) have studied the fungitoxicity of Ferbam (Fe-DMDT). By labeling with S^{35} , they found that Ferbam easily gave off a volatile compound, identified as carbon disulfide, and thus substantiating the earlier findings by Cox, Sisler and Spurr (1951), and Lopatecki and Newton (1952). The carbon disulfide was absorbed by higher plants, which could utilize the sulfur of the compound. The decomposition of Ferbam was accelerated by sulfhydryl compounds. Of these, glutathione (GSH) and cysteine were shown to reverse the effect of Ferbam, as spores of *Neurospora sitophila* and *Monilinia fructicola* immersed in a Ferbam solution for some minutes were completely inhibited when transferred to water, whereas they recovered to some extent when transferred to a solution containing a small amount of glutathione or cysteine. Believing that the effect of Ferbam may be connected with the sulfhydryl balance in the protoplasm, Weed et al tried the effect of Ferbam on urease, as representative of the SH- enzymes. At a Ferbam con-

centration of $2.5 \cdot 10^{-2}$ mg/ml, urease was partially inhibited at a concentration of $4 \cdot 10^{-5}$ mg/ml, and completely at $4 \cdot 10^{-7}$ mg/ml.

Bodine and Fitzgerald (1948) have studied the effect of Na-DMDT on the oxygen uptake by the embryo of the grasshopper, *Melanoplus differentialis*, and found that low concentrations stimulated, and high concentrations first inhibited, then markedly stimulated the respiration. The effect of copper salts was strongly counteracted by Na-DMDT. They also (Bodine and Fitzgerald, 1949) treated embryos of *Melanoplus differentialis* with Na-DMDT, whereafter they homogenized and extracted with iso-amyl alcohol. The extinction curve showed the presence of Cu-DMDT in the extract. The same authors also remark that carbon disulfide is liberated from Na-DMDT by dilution.

Analysis of plants sprayed with Fermate (=Ferbam, Fe-DMDT) or Zerlate (Zn-DMDT) shows changes in the chemical composition. This indicates that also in higher plants these substances influence the metabolism. Thus Mustard and Lynch (1945) have found that mangoes (*Mangifera mango*), sprayed with Fermate against anthracnose, contained significantly more ascorbic acid than unsprayed fruits.

Fults et al (1951) found that the amount of glutamic acid in sugar beets increased after they were sprayed with Zn-DMDT, and in a later work Blouch, Payne and Fults (1952) have studied the effect of Zn-DMDT on the amino acid content of the leaves of sugar beets. They found that the glutamic acid content decreased considerably, whereas glutamine increased. The amount of valine and leucine also increased. Tyrosine, which could not be demonstrated on the controls, gave weak spots in the treated samples. The authors concluded that treatments with Zerlate induces a metabolic shift, which among other things, results in a decrease in the glutamic content of the leaves, and an increase in the roots of the sugar beets.

Andreva and Zubkovich (1950) found a drop in photochemical activity following addition of Na-DEDT to isolated chloroplasts. The finding is later confirmed (Andreva and Zubkovich, 1953), and they stated that hydroxylamine, sodium azide and Na-DEDT, but not iodoacetate and fluorides, affected the photochemical activity of the chloroplasts. Arnon and Whatley (1954) have recently published an investigation regarding the metabolism of isolated particles from photosynthetic tissues. They found that an oxidation of oxalate occurred in suspensions of such particles. Na-DEDT inhibited relatively weakly the oxygen uptake, but quite strongly the carbon dioxide evolution.

While dealing with plants, the studies of phosphate uptake by young wheat plants in the presence of Na-DEDT made by Butler (1953) should be mentioned. His experiments show that Na-DEDT inhibits the phosphate uptake in the same manner as respiratory poisons like KCN or phosphorylating poisons like 2,4-dinitrophenol (DNP). The concentration used by him, one milli-

mole per liter, will inhibit copper oxidase completely, and even cytochrome oxidase slightly — if a similar concentration is obtained within the cell. The concentration is too high for the results to have a direct bearing on the possible fungicidal mechanism of Na-DEDT. Butler noted that Na-DEDT coloured the root cells brown. This is certainly due to formation of Cu-DEDT.

The manner in which TMTD and TMTM inhibit the growth of fungi has been virtually unknown. It has been suggested that TMTD could be reduced to DMDT ions, which in their turn could act as copper binders, but as TMTD is toxic at lower concentrations than Na-DMDT, the theory has not been considered satisfactory. Furthermore, Manten, Klöpping and van der Kerk (1951) have found that the fungicidal effect of TMTD was not counteracted by heavy metal salts.

TETD (Antabuse, Aversan) has aroused interest in medical circles, as it has been found to make the human organism sensitive toward alcohol (Hald, Jacobsen and Larsen, 1948, Hald and Jacobsen, 1948 b). Because of this it has been the object of a number of clinical and biochemical investigations. It may be considered as proven that it inhibits the aldehyde-oxidizing systems in the liver (Hald and Jacobsen, 1948 a, Larsen, Jacobsen and Hald, 1949, Newman, 1950, Newman and Petzold, 1951). There is, however, some lack of clarity regarding these enzyme systems. According to Schlenck (1951 a), there is one aldehyde dehydrogenase (aldehyde oxidase) which needs DPN (coenzyme 1) as coenzyme, but there are also one or two flavoproteins which have similar activity. These (xanthine oxidase and aldehyde oxidase) do not need DPN. Whether these really are two separate enzymes, or only one, remains unclear (Theorell, 1951). In addition, phosphoglycerol aldehyde dehydrogenase may also oxidize acetaldehyde. Which enzyme it is that oxidizes acetaldehyde *in vivo* is not known with certainty, although most evidence is in favour of the DPN enzyme.

Graham (1951) studied the inhibition of liver aldehyde dehydrogenase (Racker's dehydrogenase) by TETD in detail, and the results shall briefly be referred to here. Generally, TETD lowers the dehydrogenase-activity in homogenized rat liver, but aldehyde dehydrogenase was by far the most sensitive. The effect on this enzyme was largely reversed by GSH. TETD acted like a competitive inhibitor, competing with DPN for the active centers in the enzyme. The inhibition was 50 per cent when the dilution of TETD was $2 \cdot 10^7$, and the concentration of DPN was $1.5 \cdot 10^{-5}$ M. The affinity of the enzyme for TETD was about 50 times its affinity for GSH, and 350 times its affinity for acetaldehyde. The inhibition could thus be counteracted by low concentrations of GSH, and, it was found, also by relatively high concentrations of ascorbic acid.

Nygaard and Sumner (1952) found in a comparison of D-glyceraldehyde

-3-phosphate dehydrogenase and liver aldehyde dehydrogenase that also the first mentioned enzyme was inhibited by TETD. The mechanism of inhibition was, however, found by them to be somewhat different from that found by Graham with aldehyde dehydrogenase, as the competition was with acetaldehyde rather than with DPN. The apparent dissociation constant was found to be $5 \cdot 10^{-6}$ M. The action of TETD was also found by them to be reversed by GSH.

In connection with this, Johnston (1953) pointed out that GSH reduces TETD to DEDT ions, and that the counteracting effect of GSH on TETD may simply be due to this. It should, however, not be forgotten that many of the aldehyde-oxidizing enzymes need GSH or cysteine as a cofactor.

Other workers (Eldjarn, 1949, 1950 a and b, Johnston and Prickett 1952) have found that TETD also in the organism is reduced, so that finally carbon disulfide is formed. The connection between this reaction and the Antabuse effect has been discussed.

Kjeldgaard (1949) has found that TETD did not inhibit xanthine oxidase from milk, nor animal glucose oxidase (which is another enzyme than the fungal glucose oxidase), triose phosphate dehydrogenase (the findings of Nygaard and Sumner just referred to contradicted this), and liver alcohol dehydrogenase. Kjeldgaard found that after reduction of the disulfide to sulfhydryl (to DEDT ions), no inhibition of aldehyde dehydrogenase occurred at concentrations below 250 ppm. He also considered the inhibition as competitive.

Richert, Vanderlinde and Westerfeld (1950) agree that TETD does not inhibit xanthine oxidase from milk. According to them, liver xanthine oxidase (liver aldehyde oxidase) consists of two enzymes, one with dehydrogenase activity, and one with oxidase activity. The first of these is inhibited by TETD, but not the second. They found further that succinic oxidase was inhibited by TETD. D-amino acid oxidase was not inhibited. Keilin and Hartree (1940) had earlier found a strong inhibition of succinic oxidase by TETD and tetrathionate (among other SH-combining compounds). The inhibition was not due to the formation of an easily reversible compound, and the compound formed did not dissociate on subsequent dilution.

In a later work, Richert and Westerfeld (1951) stated that xanthine oxidase was inhibited 40—100 per cent by Antabuse, but that the inhibition was counteracted by methylene blue.

David (1951) found that the oxygen consumption, the anaerobic glycolysis and the dehydrogenase activity of yeast were considerably reduced by TETD. The concentration of TETD used, 1000 ppm., was, however, about a hundred-fold that causing complete inhibition of the growth of fungi.

Because much of the work on animal and plant enzyme systems has been

done with the ethyl derivatives, it is important to note the investigations by Kirchheim (1951), which show that TMTD is a more potent inhibitor on the aldehyde-oxidizing system in animals than is TETD. The reason why TETD is chosen, is that it has a less general toxicity.

Dale (1953) found that the esterase activity in the liver of patients treated with Antabuse fell to a very low value, while the catalase activity was unchanged. Edwards (1949) reported that the oxygen consumption of rat liver homogenates was inhibited to about 85 per cent by TETD.

Antabuse also affects the thyroid activity, possibly due to inhibition of thyroxine synthesis. Wase and Christensen (1954) supposed that the inhibition was due to the formation of a complex between TETD and iodine. They studied the formation of such a complex in vitro, and found it to have an absorption maximum at 3600 Å. Earlier, Ambrus, Ambrus and Harrison (1951) had found Na-DEDT also to possess antithyroid activity, and they ascribed it to the copper-binding properties of the compound.

Returning now to microorganisms, it should first be mentioned that with thiuram disulfides, as with the monosulfides, the oxygen consumption is only affected at concentration far above those inhibiting the growth completely (Klöpping, 1951). Sisler and Cox (1954) found that the respiration of conidia of *Fusarium roseum* in the presence of glucose was inhibited by TMTD in concentrations of 10^{-4} M or more. (Growth inhibition is caused by 10^{-6} to 10^{-5} M.) TMTD was decomposed by the fungal cells into a volatile toxicant which was probably carbon disulfide. Balestrieri (1952) found that Antabuse (TETD) inhibited the gram-positive *Staphylococcus aureus* at concentrations below 0.1 ppm (0.4 μ moles per liter), whereas the gram-negative *Escherichia coli* required a concentration 150 times as high. He stated that the inhibition was mediated by an inhibition of respiration, which was produced immediately. Combination of TETD with penicillin was found to increase the potency of both agents. According to his findings, the mechanism of action has to be somewhat different on bacteria and on fungi.

Finally, it might be mentioned that both Na-DEDT and TETD have been found to have anti-oxidant properties (Sandell, 1950, Tollenaar, 1951).

Regarding the thiuram monosulfides, no theories about the mechanism of action have been found in the literature, except that of the Dutch group, (Klöpping and van der Kerk, 1951, Klöpping, 1951, van der Kerk and Klöpping, 1952, van der Kerk, 1953), which ascribes the action of all dithiocarbamyl compounds to a special molecular configuration (see later). This configuration should also be found in TMTM.

There is a remarkable similarity of antifungal action among the different dithiocarbamyl derivatives. Manten, Klöpping and van der Kerk (1950) and Klöpping (1951) have compared the action of a large number of compounds

on fungi, and classified them according to their fungal »spectrum«. Na-DMDT, TMTD and TMTM (together with the corresponding ethyl compounds) are placed by them in group No. 1, which is characterized by the fact that *Botrytis cinerea* and *Penicillium italicum* (parasites) are about equally sensitive, further that parasites are more sensitive than saprophytes, and that among the saprophytes *Aspergillus niger* is less sensitive than *Rhizopus nigricans*. The unsubstituted dithiocarbamates and the bisdithiocarbamates (containing a diamine) belong to other groups.

A strange phenomenon which is shown by a number of fungi or bactericidal substances, but is particularly pronounced with TMTD, is the so-called inversion effect, first demonstrated by Dimond, Heuberger and Stoddard (1941). The effect occurs as a reduction of the toxicity at an intermediate concentration, on both sides of which the growth inhibition can be nearly complete. The phenomenon has been tentatively explained as resulting from dissociation of the substance to free radicals at decreasing concentrations. Montgomery and Shaw (1943) stated that the phenomenon must be ascribed to the particular chemical reactions of the compounds showing the inversion effect, as the most diverse organisms behaved similarly. Blackith (1948) assumed that there does not necessarily have to be a linear relationship between the accumulation of a substance and its concentration in the substrate, and tried to explain the effect that way.

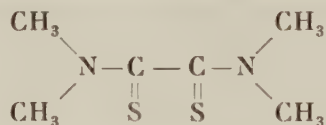
For all dithiocarbamates, the spores are more sensitive than the mycelium (Sijpesteijn and van der Kerk, 1952. Klöpping and van der Kerk, 1951). Also, Manten, Klöpping and van der Kerk (1951) have found that *Aspergillus niger* is far more sensitive to TMTD when grown on an inorganic substrate with saccharose (0.1 ppm. inhibited completely) than when grown on malt agar (10 ppm.) or on peptone-glucose agar (30 ppm). They explain this as the result of substances in the last two substrates counteracting TMTD. This has been further studied by Sijpesteijn and van der Kerk (1952), who found that L-histidine did slightly antagonize TMTD and related substances (Na-DMDT and TMTM) at higher pH values. L-histidine did not antagonize ethylene bisdithiocarbamate and tetramethylene diisothiocyanate. From these findings they concluded that the dithiocarbamyl compounds affect the histidine metabolism in the fungi.

In a later publication, Sijpesteijn and van der Kerk (1954 b) have shown that besides L-histidine, a number of other imidazole derivatives are able to antagonize the effect of Na-DMDT on spore germination in the first zone of inhibition. By a histidinedeficient mutant of *Aspergillus niger*, they were further able to prove that the antagonistic activity of the different compounds was not due to conversion to L-histidine. In addition they found that for *Aspergillus niger*, α -keto acids were active Na-DMDT antagonists in the

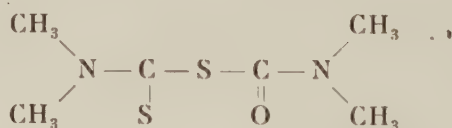
first zone of inhibition, although they were totally ineffective for the other test moulds tried, namely *Penicillium italicum*, *Botrytis allii* and *Rhizopus nigricans*.

Much work has been done in comparing the toxicity of different organic sulfur compounds. Studies have been made by Davies and Sexton (1946), and by Miller and Elson (1949). The last-mentioned authors studied the bactericidal effect, and found that towards bacteria TMTD was the most effective, followed by Na-DMDT, while TMTM was only slightly toxic. Klöpping (1951) Klöpping and van der Kerk (1951) stated as later also Palmer, Greenlee and Baldwin (1952) that among the dithiocarbamates and thiuram disulfides, the tetramethyl derivatives are the most efficient. The effect decreases with increasing chain-length in the amines. The unsubstituted or mono-substituted compounds are also considerably less toxic.

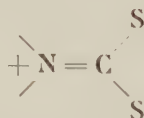
From the results of the van der Kerk group we may summarize that the methyl-ester of H-DMDT is only slightly toxic. The same holds true for Na-dimethyl thiocarbamate, and tetramethyl dithio-oxamide:



Tetramethyl thiuram oxide was, however, as toxic as TMTM. Recently, White (1954) has shown that thiuram oxides have the structure of thiocarbamyl carbamyl sulfides, and not thiocarbamyl oxides. The proper formula is thus:



Na-dithioacetate was also found to have a considerable toxicity. Klöpping and van der Kerk believed that the structure



leads to strong fungicidal activity by the dithiocarbamates and their derivatives, but not by the bisdithiocarbamates, which as mentioned have another fungicidal spectrum. For recent work on the mode of action of this group of compounds, we may mention Barratt and Horsfall (1947), Ludwig, Thorn and Miller, (1954), and Sijpesteijn and van der Kerk (1954 a).

In their paper, Sijpesteijn and van der Kerk (1954 b) discussed more generally the mode of action of dithiocarbamates, and it is felt justified to end this literature review by citing some sections of the discussion, since it represents the most recent opinions seen on this theme:

— »Nothing is known as yet concerning the actual site of action of Na-DMDT in the cells, and it seems difficult at present to understand the function of imidazoles as Na-DMDT antagonists. Mere chemical interaction between the fungicide and the various antagonists seems most unlikely. . . . The facts presented up to now rather point to the hypothesis that the imidazoles in some way or other prevent the fungicide from interaction with some essential enzyme system. . . . In the second zone of inhibition the addition of an idimazole compound alone is not sufficient to restore growth. Though *B. allii* and *Rh. nigricans* do not show a zone of inversion growth, yet here too there is clearly a first zone of inhibition in which imidazole derivatives, especially imidazole pyruvic acid, show antagonistic activity followed by a second zone in which imidazole derivatives are inactive.

»It seems logical to assume that with increase of Na-DMDT concentration gradually more enzyme systems become inhibited and thus the second zone of inhibition may well be due to the inhibition of a second essential enzyme system being superimposed on the inhibition leading to the first zone.

»With regard to the zone of inversion growth we assume that a third action of Na-DMDT, namely the blocking of a non-essential enzyme system, leads to the accumulation of an antagonist for the first inhibition zone. This antagonist might then be an imidazole derivative or, in the case of *A. niger*, an α -keto acid.»

It is definitely not easy to draw conclusions regarding the mode of action of the dithiocarbamyl compounds from the various experimental results that are reported in the literature. Some points may, however, be stressed. Thus, it might be questioned whether the dithiocarbamates act as heavy metal binders. It seems more reasonable to believe that it is the heavy metal complexes that carry the fungicidal action. It should be noted that none of the previous investigators have studied the reactions of, for example, Na-DMDT, when added to a nutrient solution, except for the formation of volatile compounds.

The structural considerations of Klöpping and van der Kerk do not explain the physiological mode of action of the compounds. Other explanations than that of a similar radical inside the molecule might even be found for the fact that the fungicidal actions of Na-DMDT, TMTD and TMTM are so similar. As these investigators cannot find any effect of the compounds on the respiratory system, they ascribe the action to the anabolic side of the metabolism. The findings of Sijpesteijn and van der Kerk that imidazole compounds counteracted the dithiocarbamyl compounds are of interest, but

one should hesitate before ascribing this to interference with a biological system. If it is remembered that L-histidine is a very powerful chelating agent, with the highest complex constants for the Cu and Zn complexes of all amino acids (Martell and Calvin, 1952), it is understood that the action may be merely chemical, consisting of a competition with the dithiocarbamates for the heavy metals.

If also the physiological action of TETD towards animal organisms is brought into the picture, it may safely be stated that not even a satisfactory working hypothesis exists regarding the mode of action of the dithiocarbamyl compounds.

In the present work, it was found of importance to start with a study of the behaviour of the various dithiocarbamyl compounds under investigation in a nutrient medium, and when in contact with cells and cellular extracts, as this seems partly to have been neglected in earlier work. First, however, various analytical methods for the compounds had to be reviewed and methods suitable for this work to be selected.

III. Analytical Methods for the Dithiocarbamyl Compounds

A number of methods for determining or assaying dithiocarbamyl compounds have been published. They have been worked out with different aims, as for instance to determine the residue on fruit crops (Dickinson, 1946, Lowen, 1951, Clarke et al, 1951). A review has been written by Patterson (1950); to determine the content of vulcanisation accelerators in rubber (Kress, 1951), or for medical or more general purposes (Domar, Fredga and Linderholm, 1949, Ferreira, 1950, Lacoste, Earing and Wiberley, 1951, Linderholm and Berg, 1951). Roughly, the methods may be divided into four groups:

To the first can be assigned those methods that use titration techniques. Na-DMDT may thus be titrated with iodine, and also in an acid-base system (cf. p. 737). TMTD can be titrated with bromate-bromide solution in chloroform (Ferreira, 1950). These methods are, however, not very specific, and not suited for the determination of the low concentrations which have to be used in physiological work.

In a second group can be placed the methods which make use of the ability of many of these compounds to give off carbon disulfide after acidification. The carbon disulfide, which is removed by aeration, is absorbed either as in the macro procedure in alkali and titrated iodometrically (Clarke et al, 1951), or it is absorbed in Viles' reagent (Viles, 1940), where the amount of Cu-DEDT formed is measured colourimetrically (Dickinson, 1946, Lowen, 1951). The last mentioned method is very well suited for microgram quantities of dithiocarbamates, as it is extremely sensitive. It has one drawback for our purpose, since it does not distinguish between the different dithiocarbamates in any degree, and not between these and carbon disulfide.

To the third group it is natural to assign the spectrophotometric methods. Spectra in the UV and visible region have been used to determine various dithiocarbamates; infra red analysis is not so suitable because of the low concentrations, and also the

low solubility of a number of these compounds in suitable solvents. For metal-DMDT compounds visible spectra have been studied by Lacoste, Earing and Wiberley (1951). As already mentioned, Koch (1949) investigated the UV spectra of some dithiocarbamyl compounds. Kress (1951) used the method for determining the presence of vulcanisation accelerators in rubber. For most of these compounds, the molar extinction values lie in the order of magnitude of 10,000/cm in the region of maximal absorption. As the various compounds have quite characteristic spectra, it is possible to distinguish between them (but not to determine quantitatively the amount of each) at a concentration level of 10 μ moles per liter. The method should thus be usable, and probably the best one, in the present work.

The fourth group of methods which also should be briefly mentioned is based on biological assay. It has been worked out for TMTD by Leben and Keitt (1950). The method is, however, not very specific, unless perhaps in combination with paper chromatography, and it is then not sensitive enough, owing to the dilution that occurs.

The spectrophotometric method of analysis was chosen for this work, partly in combination with the determination of carbon disulfide in Viles' reagent, as carbon disulfide has no characteristic spectrum with sufficiently high molar extinction values.

The dithiocarbamyl compounds have been extracted from the aqueous phase with carbon tetrachloride (tetra) and the spectrum taken up in this solution, either (for single extinction values) with a Bechman model B or model DU spectrophotometer, or, for absorption curves, with a Cary recording spectrograph. Normally, a single extraction only was performed, although it is known that this will not give quantitative extraction. The ratio water/tetra was, however, not very different in the various extractions, and in a mixed solution of the various compounds, the analytical results cannot be given as more than estimates in any case. Tetra is not the best solvent for these investigations, as it blacks out below 2600 Å, and thus only the upper part of the UV region can be used. No other suitable solvent was, however, obtainable when the investigations started. The carbon tetrachloride was ordinary technical grade, distilled once before used. As for all work where absorption in the UV region is to be measured, a high degree of cleanliness is needed. Very confusing results can be obtained in such work if the solvent during the extraction is allowed to come into contact with rubber stoppers, as these may contain Zn-DMDT and TMTD as vulcanisation accelerators, easily extractable from the rubber by organic solvents in amounts giving extinction values nearing one in the maximal region!

Some problems arise concerning the standardisation of the extinction curves for Cu-DMDT and Zn-DMDT. It is most convenient in this work, but not very feasible, to use Na-DMDT as a standard, by adding to a solution of this an excess of Zn or Cu sulfate, and extracting with tetra. This cannot, however, give sufficiently accurate results, because of the instability of Na-DMDT both in solution and as a solid substance.

To obtain a correct molar extinction value for Cu DMDT at the maximum around 4350 Å, two methods were used. First, a standard solution of cupric sulfate was prepared, and the molarity checked by electrolysis. To a measured volume of this solution was added an excess of Na-DMDT, and the Cu-DMDT formed was extracted by tetra in the usual way. Precautions had to be taken against introducing traces of copper. The extinction value was measured against a blank, prepared by extracting a solution of Na-DMDT only. Only the value at 4350 Å was determined in this

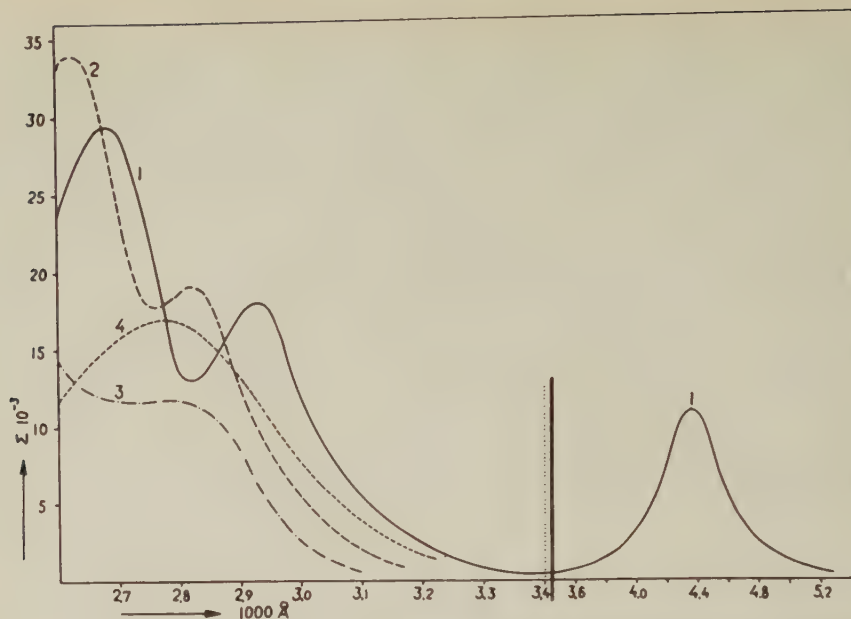


Figure 1. Absorption spectra of Cu-DMDT (curve 1), Zn-DMDT (curve 2), TMTD (curve 3), and TMTM (curve 4). Ordinate: molar extinction value per cm.

manner, as it was feared that a less reliable spectrum in the UV region could be obtained when an excess of Na-DMDT was used, owing to the traces of TMTD always found in Na-DMDT. The other method used was to prepare a standard solution of Cu-DMDT by dissolving in pyridine a weighed amount (recrystallized from pyridine), and diluting it with alcohol to a concentration which could be measured. The results with these two methods agreed well, the values obtained lying between 10,500 and 11,500. In this work, the molar extinction value of Cu-DMDT at 4350 Å is therefore considered to be 11,000. This is a surprisingly high value when seen in the light of attempts to determine it from standard solutions of Na-DMDT, by adding cupric sulfate and extracting. The extinction values then obtained were very variable, but higher values than 8500 per two moles of Na-DMDT were very rare.

The spectrum of Zn-DMDT was obtained by adding an excess of Na-DMDT to a known amount of zinc sulfate, extracting and correcting the spectrum for Cu-DMDT by means of the extinction value at 4350 Å.

For TMTD and TMTM, the problem of standardisation is quite simple, owing to the stability and crystallinity of these compounds. In figure 1 are seen the spectra of Zn-DMDT, Cu-DMDT, TMTD and TMTM in tetra. Figure 2 shows the spectrum of Na-DMDT in water.

The spectra of the higher dialkyl derivatives were found to be very similar to the dimethyl derivatives, but the unsubstituted dithiocarbamates have different spectra. Cu-DT was investigated, and found to be quite featureless in the near-UV region. This compound was also practically insoluble in tetra. As the unsubstituted compounds also are unstable in water and result in other decomposition products than

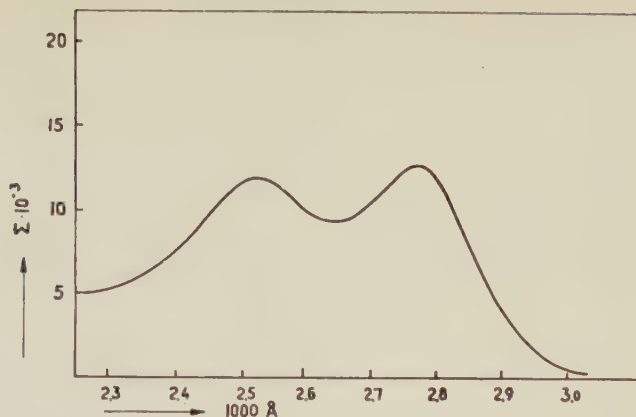


Figure 2. Absorption spectrum for Na-DMDT in water. Ordinate: Molar extinction value per cm.

the dialkyl derivatives, their analysis leads to quite other problem than do the dialkyl derivatives. This is not taken up in this work.

IV. Studies on the Stability of the Dithiocarbamyl Compounds, and their Behaviour in Biological Systems

A. Reactions in a Mineral Salts Nutrient Solution

It is well known that the dithiocarbamates hydrolyze to give carbon disulfide and amine salt in acid solution (cf. preceding chapter). The stability of Na-DMDT at various pH values has been investigated by Lopatecki and Newton (1952), and that of Fe-DMDT by Weed et al (1953).

A titration curve for Na-DMDT with hydrochloric acid is seen in figure 3. It was observed during the titration that the pH drifted towards higher values after each portion of acid added, and became stable after about five minutes. This drift can partly be caused by the decomposition of H-DMDT, and partly by the reaction of the liberated amine with acid. It is seen from the curve that two equivalents of acid were needed to reach the end point. When the solution afterwards was back titrated with sodium hydroxide, it behaved like a strong acid-base system, with only the excess acid added still present. This proves the complete decomposition of H-DMDT with formation of carbon disulfide and dimethyl amine hydrochloride.

In an unbuffered system, the hydrolysis of Na-DMDT will induce a shift towards higher pH values, and this will help in stabilizing a moderately concentrated solution of Na-DMDT. In a buffered system, the stability of the compound will (when complexing cations or oxidizing agents are absent) be strongly dependent on pH. The results from an experiment demonstrating

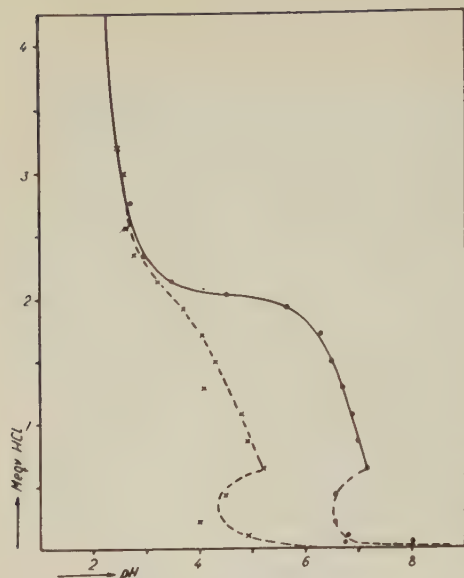


Figure 3. *Titration curve for Na-DMDT. 1 milliequivalent titrated with 0.1-n HCl. pH of the Na-DMDT-solution at start was about 10. Circles and fully drawn line, the pH values obtained 5 minutes after the addition of acid. Crosses and dotted line: pH values noted immediately after the addition of acid.*

this are shown in figure 4. It is seen that at pH 3, the half life (this expression not taken in the strict mathematical sense, as no proof for a first order reaction has been presented) is only about fifteen seconds, whereas at pH 4.7, it is a little less than thirty minutes. At pH above 6, the half life was found to be considerably longer, of the order of magnitude of a couple of days. *From this it can be concluded that in experiments with these compounds, the pH of the solution should not be allowed to drop below 6.*

In biological investigations and particularly in growth experiments, it is, however, virtually impossible not to introduce heavy metals to some extent, and therefore the free dithiocarbamate ion concentration will also depend on the concentration of these. Especially in an ordinary nutrient solution will this be the case, as is shown in the following experiments.

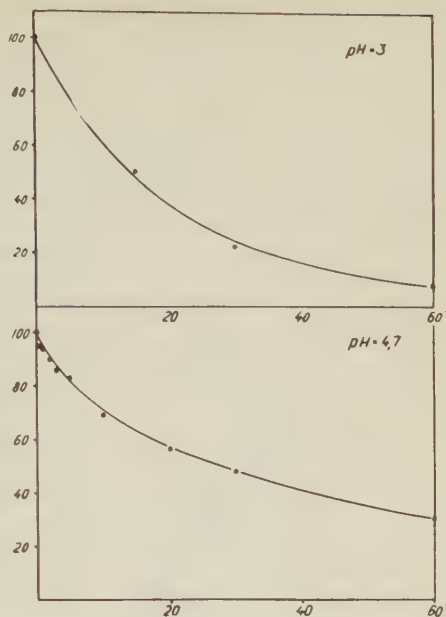
As a ›model‹ nutrient solution was used a Czapek medium made up from the following stock solutions:

1. Mineral solution without N and P

KCl	12.5 g
MgSO ₄ · 7H ₂ O	12.5 g
FeCl ₃	0.25 g
ZnSO ₄ · 7H ₂ O	0.25 g

Tap water to 10 liters. (The municipal tap water used is very soft and has a low dry matter content, but contains somewhat high amounts of copper from the pipes in the laboratory building.)

Figure 4. The stability of Na-DMDT at pH 3 and at pH 4.7. The start concentrations were approximately 100 μ M. The remaining DMDT ions in the phosphate buffer were determined by transferring samples at suitable time intervals to neutral buffer solutions of known volume, with a little cupric sulfate added. The amount of Cu-DMDT formed was then measured spectrophotometrically. The experiments were made at room temperature. Ordinate: remaining DMDT ions, percentages of the start concentrations. Abscissa: upper curve, time in seconds; lower curve, time in minutes.



2. N-solution

NH_4Cl 26.8 g
Distilled water to 5 liters

3. Phosphate buffer

Mixtures of 0.25 M KH_2PO_4 and K_2HPO_4 (in distilled water), to the desired pH.

4. Carbohydrate solution

Glucose 125 g
Distilled water to 5 liters.

The nutrient solution was prepared by mixing four parts of 1 with two parts each of 2 and 3. This mixture was heated to boiling and carefully filtered, whereafter two parts of 4 were added. The pH value used was 7. In the experiments reported here, the nutrient solution was not further sterilized.

In the first of these experiments, varying amounts of Na-DMDT were added to nutrient solutions in 300 ml. erlenmeyer flasks. (In this connection it was found necessary first to purify the Na-DMDT, which had been prepared a couple of months earlier and stored in a closed bottle at room temperature. On extraction with tetra, it was found to contain TMTD. This could be removed by repeated extractions of an aqueous solution with tetra.)

From the flasks samples were extracted with tetra immediately after preparation. Then the flasks were incubated for 24 hours at 30° C, when new samples were extracted.

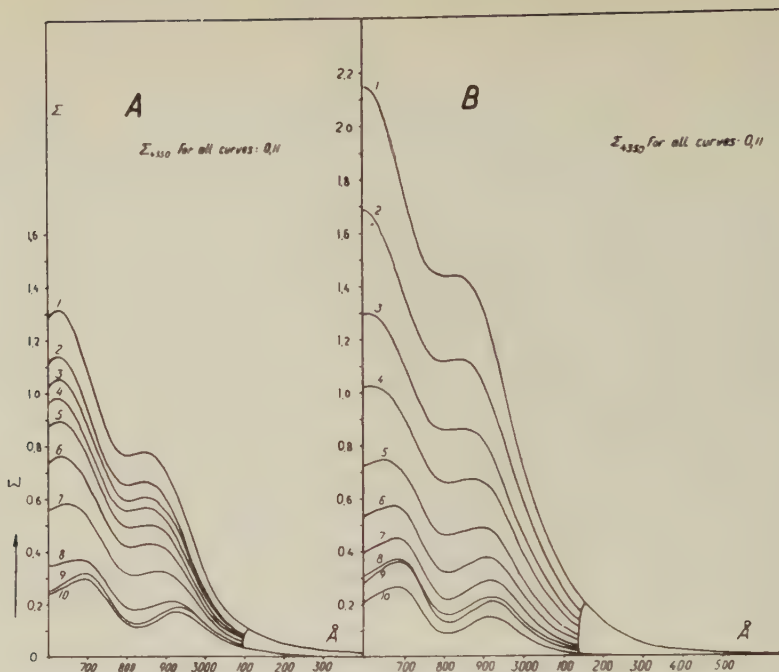


Figure 5. Absorption spectra of extracts of nutrient solutions to which have been added varying amounts of Na-DMDT. Curves in A from samples extracted immediately; curves in B from samples extracted after incubation for 24 hours at 30° C. The numbers 1—10 on the curves represent the following Na-DMDT concentrations: 500, 300, 200, 150, 100, 70, 50, 30, 20, 10 μ moles per liter, respectively.

The absorption spectra of the tetra extracts are shown in figure 5. It is seen that a tetra-soluble compound has appeared during the incubation. Most probably this is TMTD, as when the 200 μ M absorption curve is subtracted from the 500 μ M one, a very TMTD-like spectrum appears.

In figure 6 A and B are shown the extinction values at three wavelengths as a function of the amount of Na-DMDT added, for the extracts taken immediately (figure 6 A), and after 24 hours (figure 6 B). These curves, seen in connection with the absorption spectra in Figure 5, show that at low concentrations of Na-DMDT, all of it is bound as Cu-DMDT. When the concentration increases, the excess combines with zinc to form Zn-DMDT. This complex is not so strong as the Cu-DMDT complex, and can from the shape of the curves in figure 6 be seen to be dissociated to some extent. But only when most of the zinc ions are bound as Zn-DMDT will the solution contain free dithiocarbamate ions to any degree, and from then on the concentration of these will be proportionate to the amount of Na-DMDT added subsequently.

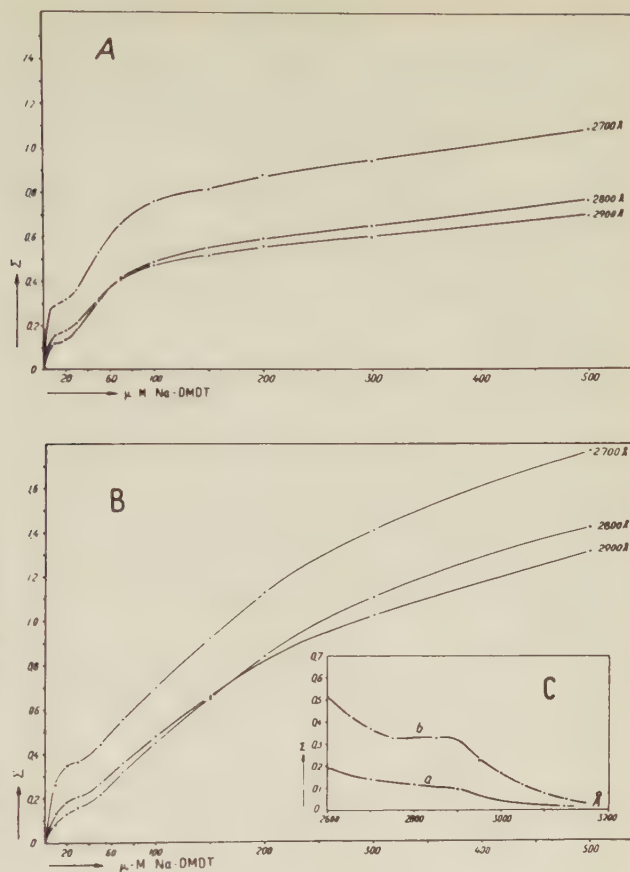
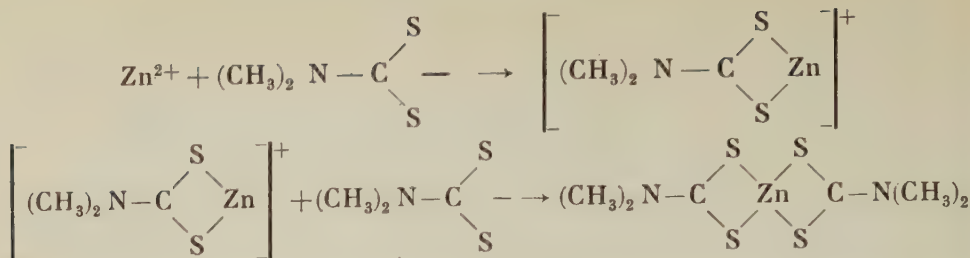


Figure 6. The extinction values from figure 5, at the wavelengths 2700, 2800 and 2900 Å as functions of the Na-DMDT concentrations. Figure A gives the results from A in fig. 5, and B those from B in fig. 5. Figure C, the difference between the spectra after addition of 200 and 500 μM Na-DMDT. Curve a from figure 5 A, and curve b from figure 5 B.

The curves of the extracts taken after 24 hours show the same general picture as those taken at the start, but a disappearance of dithiocarbamate during the incubation has resulted in a stretching of the curves along the abscissa in figure 6 B. Roughly half the amount of Na-DMDT added must have disappeared, but only a minor amount seems to have been oxidized to TMTD. About 32 μM Na-DMDT, or 10 per cent of the amount present at the start, has been oxidized between 200 and 500 μM Na-DMDT (curve 6 C).

No metal complexes other than Cu-DMDT and Zn-DMDT seem in this case to be formed with the dithiocarbamate. Probably the nutrient solution, because of the boiling and filtering, contained only a very small amount of iron.

Zn-DMDT and Cu-DMDT will establish reversible dissociation equilibria in the solution; for Zn-DMDT the following equations may be written:



If the dithiocarbamate ion is denoted X^- , the stability constants are expressed thus:

$$\frac{C_{\text{ZnX}^+}}{C_{\text{Zn}^{2+}} \cdot C_{\text{X}^-}} = k_1$$

$$\frac{C_{\text{ZnX}_2}}{C_{\text{ZnX}^+} \cdot C_{\text{X}^-}} = k_2$$

$$\frac{C_{\text{ZnX}_2}}{C_{\text{Zn}^{2+}} \cdot C_{\text{X}^-}^2} = k_1 k_2$$

Similar equations may be written for Cu-DMDT. In order to know the relative concentrations of the different complexes, the values k_1 and k_2 should be known. No stability constants for dithiocarbamate complexes have been found in the literature. Because of the low solubility of the 1:2 complexes, and the instability of the free dithiocarbamic acid, the constants are difficult to determine.

In the present work, attempts have been made to obtain only rough estimates, so that at least a qualitative picture of the degree of dissociation of the complexes at the concentration range used in the biological investigations could be given.

An approximation of the value $k_1 k_2$ for Zn-DMDT can be obtained from the curves in figure 6 A, although the extraction procedure might have caused a shift in the equilibrium. Data for the calculation are found in table 1. Attention should also be called to table 4, where values are given for the amount of Zn-DMDT extractable from solutions (or centrifuged suspensions) of different concentrations. From these values, $k_1 k_2$ values of $7.5 \cdot 10^7$, $2 \cdot 10^8$ and $3.4 \cdot 10^9$ respectively, going from the highest concentration to the lowest, can be calculated. The first value is not reliable, because Zn-DMDT at the highest concentration certainly had precipitated. The two lower values agree as regards the order of magnitude with those obtained from figure 6 A.

It should be noted that in these calculations it has been assumed (1) that all Zn-DMDT (1:2) formed is extracted by tetra without a shift being produced in the equilibrium, and (2) that the remainder in solution exists as free zinc and dithiocarbamate ions. Neither of these assumptions holds true.

As only a part of the Zn-DMDT (1:2) which is formed is extracted, and equilibria will be established between the concentrations of Zn-DMDT (1:2) in the tetra and in the aqueous phase and Zn-DMDT (1:1), Zn^{2+} and DMDT-

Table 1. Estimation of the stability constant $k_1 \cdot k_2$ for Zn-DMDT by means of data from the curve for 2900 Å in figure 6 A. It is assumed that all Zn-DMDT (1: 2) has been extracted, and that the remainder is completely dissociated. See the text for a discussion of the method. The concentrations are given in the table as moles per liter. 10^6 .

$C_{\text{Na-DMDT}}$	0	5	15	35	55	85	123
$C_{\text{Zn-DMDT}}$	0	0.3	1.3	4.8	9.45	10.35	10.7
$C_{\text{Zn}^{2+}}$	10.7	10.4	9.4	5.9	1.25	0.35	0
C_{DMDT^-}	0	4.4	12.7	25.4	35.1	64.3	123.6
$\log C_{\text{Zn-DMDT}}$..		— 0.52	0.11	0.68	0.98	1.02	
$-\log C_{\text{Zn}^{2+}}$...		— 1.02	— 0.97	— 0.77	— 0.11	— 0.46	
$-2 \log C_{\text{DMDT}^-}$		— 1.28	— 2.22	— 2.80	— 3.11	— 3.62	
$\text{pk}_1 \cdot k_2 - 12$		— 2.82	— 3.08	— 2.89	— 2.13	— 2.16	
$\text{pk}_1 \cdot k_2$		9.18	8.92	9.11	9.87	9.84	

in the aqueous phase, it is not possible to calculate the value $k_1 k_2$ with any accuracy without knowing the distribution coefficient of Zn-DMDT (1:2) between tetra and water, and the concentration of Zn-DMDT (1:1) in the aqueous phase. Since the distribution coefficient certainly is higher than 1 in the present case, but below, for example, 100, the error introduced by omitting the amount of Zn-DMDT (1:2) remaining in the aqueous phase from the calculation will not be so serious. But by assuming that instead of zinc and DMDT ions, the aqueous phase contains Zn-DMDT (1:1) and DMDT ions, it is seen that the calculated coefficient will be k_2 , and not $k_1 k_2$. From the preceding, we can therefore only conclude that the true value for $k_1 k_2$ lies between 10^9 and $10^9 \cdot k_1$.

Attempts to obtain values for k_1 were made in two directions. First, absorption spectra of dilute aqueous solutions of zinc sulfate with Na-DMDT added demonstrated a considerable dissociation (figure 7). It is seen that when 10 μM zinc sulfate and concentrations of Na-DMDT up to 15 μM are mixed, the spectrum obtained closely resembles that of Na-DMDT alone. Only a slight increase in the value $\epsilon_{2540}/\epsilon_{2788}$, from 0.93 for Na-DMDT to 1.00 for the mixture, was observed. The maxima are seen still to be at the wave-lengths for Na-DMDT. When the zinc concentration is increased to 1000 μM , a spectrum resembling that of Zn-DMDT in tetra is seen to appear. The maxima are at 2480 and 2720 Å, and with a relative ratio $\epsilon_{2480}/\epsilon_{2720}$, = 1.25.

It can now be concluded that k_1 must be of the order of magnitude of 10^4 . If k_1 is 10^5 , only 50 per cent of the Zn-DMDT (1:1) complex would be dissociated in the concentration range of 10 μM , and the spectrum would then show characteristics of the 1:1 complex in a much higher degree. If k_1 is

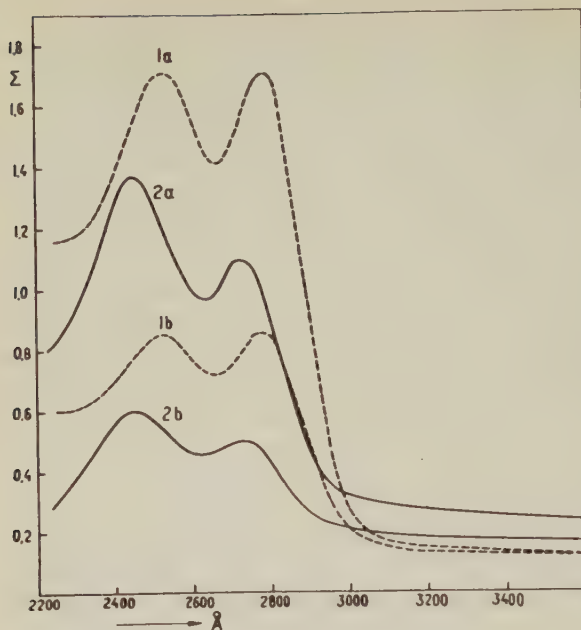


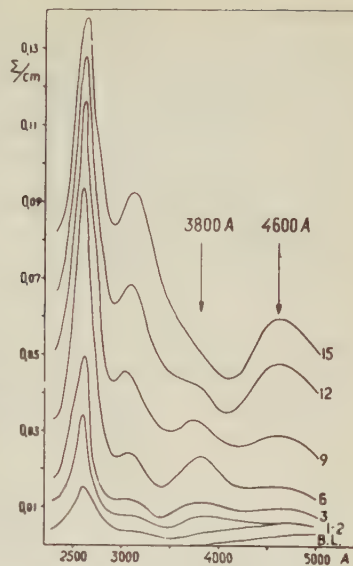
Figure 7. Absorption spectra of aqueous mixtures of Na-DMDT and zinc sulfate. The curves were taken with Cary recording spectrophotometer, using 10 cm cuvettes. Curves 1, with 10 μM zinc sulfate; Curve 1 a, with 15 μM Na-DMDT and curve 1 b, 7 μM Na-DMDT. Curves 2, 1000 μM zinc sulfate, 2 a with 15 μM Na-DMDT and 2 b with 7 μM Na-DMDT. The blank cuvettes contained the corresponding amounts of zinc sulfate alone.

10^4 , 10 per cent would exist as the 1 : 1 complex in this concentration range, and only a slight shift in the spectrum should be visible. When the zinc ion concentration is increased to 1000 μM , practically all Na-DMDT added (when in a concentration range of 10 μM) would be converted to Zn-DMDT (1 : 1), even if k_1 were as low as 10^2 . The spectra obtained under these conditions are thus most likely the spectra of the pure 1 : 1 complexes.

These considerations were checked by polarographic studies. When a Sargent polarograph with a dropping mercury cathode and a saturated calomel reference anode was used, a 20 μM zinc sulfate in 0.25 M potassium phosphate buffer of pH 6.3 gave a wave at -1.11 volts, 20 mm in height. After addition of 12.5 μM Na-DMDT, the zinc wave decreased to 17 mm, and another wave at 1.33 volts appeared. This wave must be ascribed to the Zn-DMDT (1 : 1) complex, according to the spectrophotometric studies. From the decrease in height in the zinc wave following the first and successive additions of Na-DMDT, k_1 can be estimated to be 10^4 .

Returning now to the value $k_1 k_2$. As mentioned above, $k_1 k_2$ must lie between 10^9 and $10^9 \cdot k_1$. Only an order of magnitude of 10 per cent of the added zinc and dithiocarbamate ions exist in the form of the 1 : 1 complex when the concentrations are in the range of 10 μM , and less than this in the form of the 1 : 2 complex, before the extraction with tetra. After extraction, the concentrations in the aqueous phase will have decreased, and a further

Figure 8. Absorption spectra of a 20 μM solution of cupric sulfate to which has been added increasing concentrations of Na-DMDT. The numbers on the curves give the concentrations of Na-DMDT in $\mu\text{moles per liter}$. B.L. means base line (blank line) for the recording instrument. It should be noted that with increasing concentrations of Na-DMDT, the solution becomes more turbid, due to precipitation of Cu-DMDT. The spectra were taken with cuvettes with 10 cm light path. Ordinate: extinction value per cm.



dissociation must be expected. The conclusion is therefore finally reached that the value obtained by the extraction procedure must be correct as regards order of magnitude, and we may in our rather qualitative estimations assume k_1 to be 10^4 , and k_1k_2 to be 10^9 .

On the basis of these roughly estimated values, the relative concentrations of free dithiocarbamate ion and of the 1:1 and the 1:2 complexes can be calculated for varying concentrations of zinc and Na-DMDT. A set of such curves is shown in figure 8.

As regards Cu-DMDT, attempts to estimate stability constants did not succeed, owing to the high values of the constants and the very low solubility of the 1:2 complex. The existence of a 1:1 complex could, however, be demonstrated very nicely by spectrophotometric technique.

A solution of 20 μM cupric sulfate in distilled water was prepared, and placed in a 10 cm Cary cuvette. As blank was used a similar cuvette filled with distilled water. To the cuvette containing cupric sulfate was then added (from a stock solution) 1 mM Na-DMDT in small amounts, to give concentrations of 1, 2, 3, etc. μM in the cuvette. After each addition, the spectrum was taken. The results are seen in figure 8. It is noted that after addition of small amounts of Na-DMDT, a peak appears at 3800 Å. This peak seems to reach a maximum when about 6 μM has been added, and at increasing concentrations it then seemingly disappears as the ordinary maximum at 4500 Å (the maximum which in tetra is at 4350 Å) appears. Since the peak

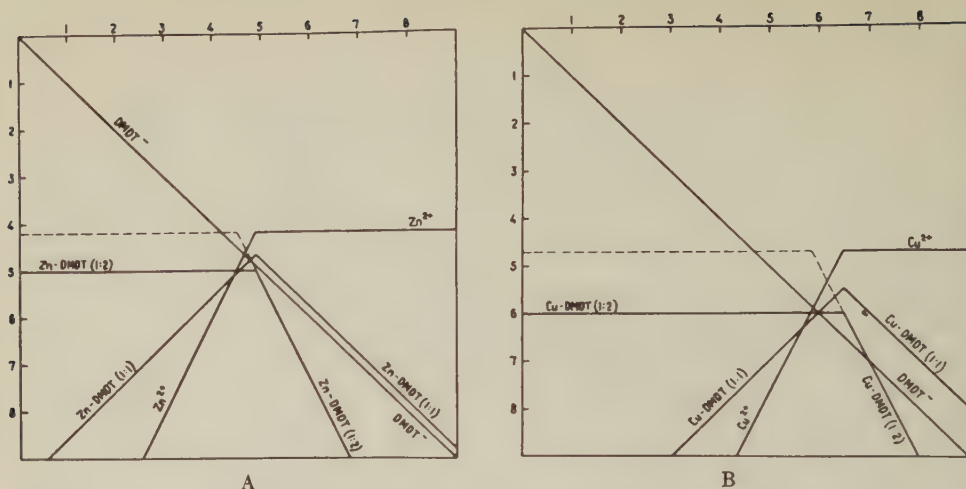


Figure 9. The relative ratio between free dithiocarbamate ion, free heavy metal ion, 1 : 1 and 1 : 2 complexes of Zn-DMDT (fig. A) and Cu-DMDT (fig. B). The abscissa denotes the negative logarithm of the concentration of the free DMDT-. The negative logarithm of the concentrations of the other compounds are found along the ordinate. The following assumptions have been made: For Zn-DMDT, $k_1=3 \cdot 10^4$, $k_2=3 \cdot 10^4$, $C_{Znso_4}=6 \cdot 10^{-5}$ M; the solubility of Zn-DMDT (1 : 2) = 10^{-5} M. For Cu-DMDT, $k_1=5 \cdot 10^7$, $k_2=10^6$, $C_{Cuso_4}=2 \cdot 10^{-5}$ M; the solubility of Cu-DMDT (1 : 2) = 10^{-6} M.

appears when cupric ions are in excess, it is very reasonable to ascribe it to the 1 : 1 complex.

When the extinction values from figure 8 were plotted against the concentration at various wave lengths, curves were obtained which showed no detectable dissociation of the complexes at the concentrations used. It is also noted that the spectra do not show the presence to a high degree of the DMDT ion. This seems to mean that k_1 must lie above 10^6 , and k_1k_2 probably above 10^{12} . It is not possible to state how high the values really are. In figure 9 are drawn a set of curves, assuming $k_1=5 \cdot 10^7$ and $k_2=10^6$.

Another problem causing difficulties is the determination of the actual solubilities of the 1 : 2 complexes. These are probably very low both for Cu-DMDT and Zn-DMDT. In table 2 are given values found by extraction of saturated solutions of Zn-DMDT and Cu-DMDT. These values do not necessarily represent the amounts of undissociated 1 : 2 complexes in solution. If the distribution coefficient for the 1 : 2 complex goes strongly in the favor of the tetra phase, a shift in the equilibrium will cause the formation of more of the 1 : 2 complexes during the extraction.

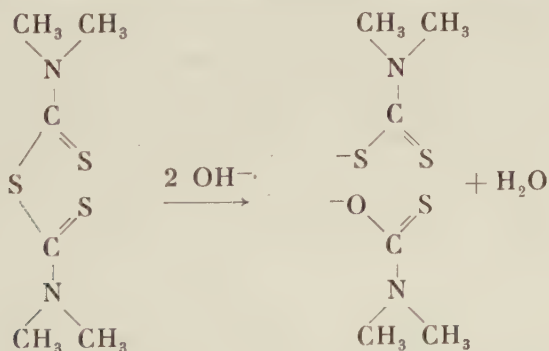
An attempt was also made to determine the solubilities by adding zinc or cupric sulfate in dilute solutions to relatively concentrated Na-DMDT, and

measuring the concentration of the heavy metal at the point where the turbidity increases from zero. Under such conditions, the concentrations of the heavy metal 1 : 2 complexes will be practically equal to the concentration of the added heavy metal. The method worked well for Zn-DMDT, as the turbidity rose sharply and practically linearly after addition of a certain concentration of zinc sulfate. With Cu-DMDT, the increase in turbidity was, however, so slight that it was very difficult to state at which concentration the precipitation had started. Results from these experiments are also found in table 2.

The solubilities of TMTD and TMTM have been assayed by extraction of saturated solutions (table 2). These compounds are considerably more soluble than the metal chelates, and the solubility of TMTM is remarkably high.

TMTD takes part in redox processes, and it was always found to be reduced to a small extent, particularly in solutions with cupric ions that could bind the DMDT ions formed. A considerable reduction can, however, not take place in an ordinary nutrient solution. In the polarographic studies reported previously, an anodic wave at -0.58 V was observed when larger amounts of Na-DMDT were present. Against a normal hydrogen electrode, the redox potential of the system TMTD/DMDT $^-$ should then be -0.34 V. Gregg and Tyler (1950) found for the system TETD/DEDT $^-$ a redox potential of -0.303 V.

TMTM can be hydrolyzed to DMDT ions and dimethyl thiocarbamate ions, the latter probably undergoing further decomposition:



Due to this reaction, TMTM is unstable in alkaline solution, but the hydrolysis will also take place in neutral solutions in the presence of cupric ions. This can be demonstrated by adding a little cupric sulfate to a solution of TMTM. The light yellow colour will then darken, and become yellow-brown from Cu-DMDT.

Table 2. Attempts to determine the solubility of Zn-DMDT, Cu-DMDT, TMTD and TMTM in distilled water, 0.1 M phosphate buffer of pH 7.0, and in nutrient solution. Solubility in μM .

Solvents	Zn-DMDT		Cu-DMDT		TMTD	TMTM
	Extraction	Turbidity	Extraction	Turbidity	Extraction	Extraction
Dist. water	7.0	5	4.4	2	110	3,300
	8.8	8	3.1		108	2,100
Phosphate buffer	29.5		2.5		104	2,000
	29.0		2.6		102	3,300
Nutrient solution	21.0		1.2		66	2,300
	27.0		1.5		86	2,200

An experiment with the intention of studying the stability of Zn-DMDT, Cu-DMDT, TMTD, and TMTM when added to the nutrient solution as such, was made in much the same manner as the experiment with Na-DMDT. Erlenmeyer flasks each with 100 ml of nutrient solution containing about 20, 50, and 100 μM of these substances respectively were prepared and samples extracted immediately. The flasks were then incubated for 24 hours at 30° C, and samples extracted anew. The extracts were run in the Cary spectrophotometer, except for Cu-DMDT, where only the extinction at 4350 Å was measured in the Beckman. The results are seen in figure 10.

It appears that only Cu-DMDT is somewhat stable. The Cu-DMDT-impurity in Zn-DMDT has kept fairly constant, but Zn-DMDT has to a large extent disappeared, probably as carbon disulfide, as the spectra do not indicate the appearance of TMTD. TMTD has also kept fairly constant, especially at the higher concentrations, but it is seen that the solutions contain quite a lot of Cu-DMDT. TMTM was from the start somewhat hydrolyzed, and contained Cu-DMDT, and this process has continued during the incubation.

The next study concerned how a nutrient solution with dithiocarbamyl compounds present behaved when *aerated*. These experiments and the subsequent growth experiments were carried out in a constant temperature bath equipped with a device for magnetic stirring of the contents of the culture flasks.

The constant temperature bath was a glass vessel about 30 by 40 cm. and 25 cm high. It was filled with mineral oil and equipped with a contact thermometer, relays and heating element. In this bath, supported by a frame of aluminium, were placed seven permanent magnets, rotating on vertical axes just below a thin plate of aluminium, upon which the culture flasks were placed. The magnets were interconnected with a perspex gear system, and placed in a pattern to allow them all to be driven by a variable-speed motor, and with space enough for a culture flask above each. The rotating gears also circulated the mineral oil in the bath, rendering a special stirring motor unnecessary.

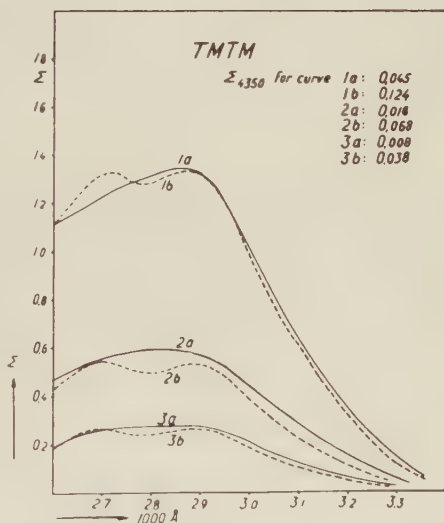
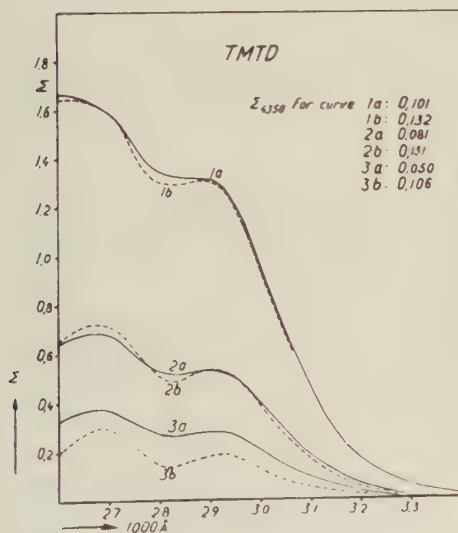
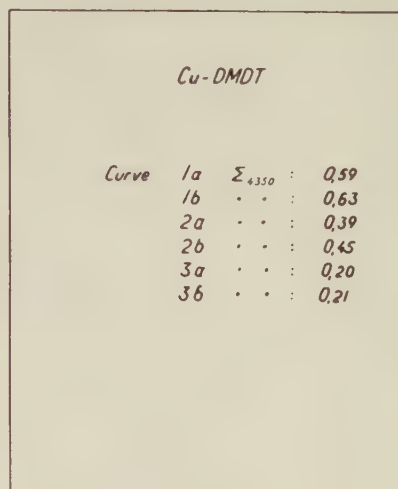
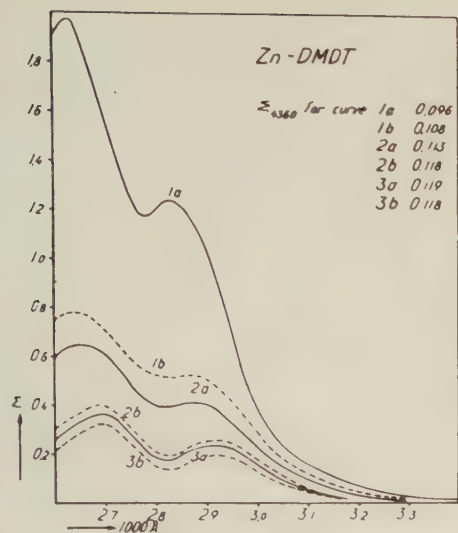


Figure 10. Absorption spectra of extracts of nutrient solutions with varying amounts of Zn-DMDT, Cu-DMDT (only extinction values at 4350 Å given), TMTD and TMTM added. Curves marked a, from samples taken and extracted immediately. Curves marked b, from samples incubated 24 hours at 30° C before extraction. Curve No. 1, 100 μ M, No. 2, 50 μ M and No. 3, 20 μ M of the dithiocarbamyl compound.

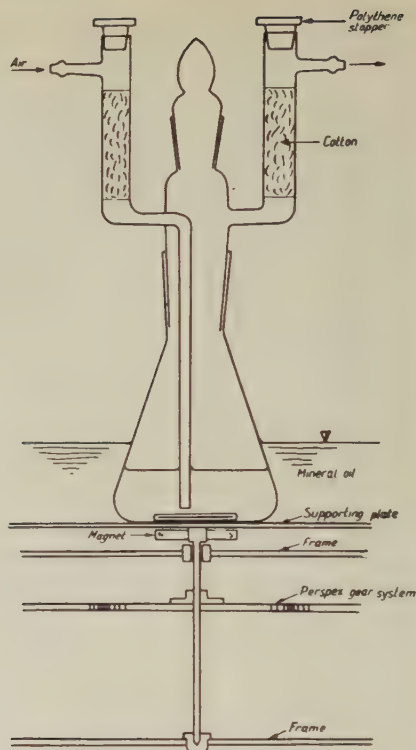


Figure 11. Culture flask with aeration and magnetic stirring.

The culture flasks were made from 300 ml Pyrex erlenmeyer flasks by substituting a ground-glass neck for the ordinary one. A flask with the magnetic stirring equipment is shown in figure 11. It should be mentioned that when the flasks are to be used for sterile work, they must be autoclaved with the polythene stoppers on the side tubes absent, as they would otherwise melt.

The flasks were aerated by means of a gassing manifold and a Medwac pump. A T-tube with a clamp served as a regulating mechanism for the main current of air, and a clamp before each culture flask regulated the air-stream through the flask. Later, the flasks were also equipped with flow-meters of the type measuring the pressure drop through a capillary, for a better control of the air flow through each flask. The air was passed through a washing bottle with lead acetate before entering the flasks, and after leaving them it passed through washing bottles with zinc acetate, to trap any hydrogen sulfide, and thereafter through measured volumes of Viles' reagent in test tubes.

Two experiments were carried out, in which portions of 100 ml nutrient solution, containing 20 μ M of, respectively, carbon disulfide (in two flasks), Na-DMDT, Zn-DMDT, Cu-DMDT, TMTD and TMTM were aerated for one hour at 30° C. Then the Viles' reagent was changed, 25 ml normal sulfuric acid was added to each culture flask, and they were aerated anew for one

Table 3. *The behaviour of the dithiocarbamyl compounds in aerated nutrient solutions.* Experiments in the constant temperature bath, with 100 ml nutrient solution, to which has been added 20 μ M of carbon disulfide (to two flasks), Na-DMDT, Zn-DMDT, Cu-DMDT, TMTD, TMTM. Temperature 30° C. A fairly rapid bubbling of air through the flasks during the whole experiment. The air was passed through 10 ml of Viles' reagent for absorption of carbon disulfide. After one hour, 25 ml *N* sulfuric acid was added to each flask, the Viles' reagent renewed, and the aeration continued for another hour. At the end of the experiment, the contents of the flasks were extracted with tetra. The amount of Cu-DMDT formed was calculated from the extinction value at 4350 Å, and the amount of other compounds formed was calculated from the UV-spectrum, taken with the Cary recording spectrophotometer.

Compound added	CS ₂ after aeration (1 hr)	CS ₂ after acidific.	Cu-DMDT in the sol.	Other compounds	Sum
CS ₂	19.9	0.1			20.0
CS ₂	19.5	0.5			20.0
Na-DMDT.....	0.0	0.85	7.50	TMTD 1.95	19.8
Zn-DMDT.....	0.3	5.8	11.2	Zn-DMDT 4.85	19.1
Cu-DMDT.....	0.0	0.0	19.8	—	19.8
TMTD	0.1	0.0	2.25	TMTD 17.8	20.1
TMTM	0.0	0.5	0.25	TMTM 18.0	19.0

hour. The two experiments gave essentially the same result, and data from one are compiled in table 3. It appears that, in neutral solution, the compounds are fairly stable towards aeration. Surprisingly enough, Zn-DMDT is less stable than Na-DMDT. This is even more clear in acid solution. No hydrogen sulfide formation was observed.

B. Reactions When in Combination with Cellular Extracts

As now the reactions of the dithiocarbamyl compounds in the nutrient solution had to some extent been elucidated, it seemed natural to turn attention to their behavior when in contact with cellular constituents, to see if some particular reactions might occur.

An informative experiment was carried out in the same manner as the previously mentioned one in the constant temperature bath and with aeration, but with an addition of acetone yeast powder to the nutrient solution, corresponding to 1 per cent dry weight in each. This experiment showed that Na-DMDT, Zn-DMDT, Cu-DMDT and TMTM behaved as without yeast extract, but TMTD was reduced. As additional experiments showed that reduction also occurred in the presence of boiled yeast extract, as well as cysteine and ascorbic acid, it can probably be considered as unspecific and non-enzymatic.

But one rather intriguing difference from the earlier experiment was found, namely that the quantitative recovery of the added dithiocarbamyl compounds was found to be very far from satisfactory. Repetition of the

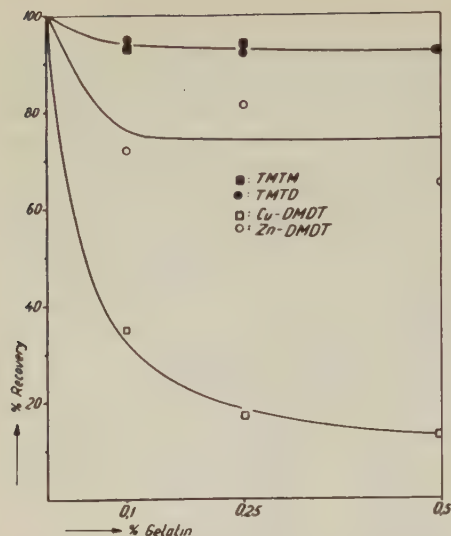


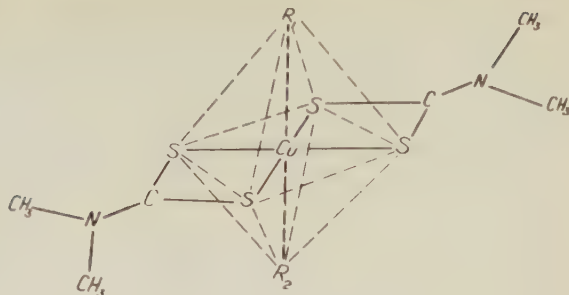
Figure 12. The recovery in the tetra phase after extraction of 80 μ M Zn-DMDT, Cu-DMDT, TMTD and TMTM from distilled water containing various concentrations of gelatin.

experiment showed that this was not due to an experimental error. In order to clear up this problem, extraction tests were made with gelatin in phosphate buffer, as a simple and nonspecific protein system, it being natural first to suspect the protein of being the cause of the discrepancy. Linderholm and Berg (1951) had earlier found that Cu-DEDT could not be extracted from serum solutions.

The results from one such test are shown in figure 12. It is seen that TMTM and TMTD are extracted satisfactorily in the presence of protein, Zn-DMDT not so well, and Cu-DMDT is practically all retained in the aqueous phase, even if this contains as little as 0.1 per cent gelatin. It was further noted, that whereas normally an aqueous suspension of Cu-DMDT of the concentration used here was distinctly turbid, the gelatin-containing suspension (or solution) remained quite clear, with no signs of precipitation. When the gelatin was precipitated with ammonium sulfate or alcohol, Cu-DMDT followed the precipitate.

The behavior of Zn-DMDT seems best explained by assuming that traces of copper in the gelatin (such could be shown to be present) have transferred some of the Zn-DMDT to Cu-DMDT. The problem rests therefore with Cu-DMDT. It is very difficult to perceive how the distribution equilibrium between the polar and the non-polar phase can be changed by the protein solution, as it seemingly is here. It is particularly difficult to understand how only the distribution equilibrium for Cu-DMDT can be affected. The only reasonable solution seems to be that a sort of complex must be established between the protein molecule and Cu-DMDT. Such a complex formation is not improbable, as the structure of Cu-DMDT is square, and

Figure 13. Hypothetical structure of a complex between Cu-DMDT and protein. R_1 and R_2 denote groups in protein molecules.



cupric complexes with the coordination number of six are known (Pfeiffer and Pimmer, 1905, Peyronel, 1941). These complexes are octahedral, and they could be formed from Cu-DMDT by two additional bonds vertical to the square, as shown in figure 13. For these two additional bonds the cupric atom has one electron-pair and one lonely electron. On this basis the unique position of Cu-DMDT can be understood. But it must be stressed that no final proof for the existence of additional bonding ability in Cu-DMDT has been given. It was attempted to prepare a di-pyridinium complex of Cu-DMDT by recrystallizing it from pyridine, but this failed. The only indication of an interaction was the comparatively high solubility of Cu-DMDT in pyridine. Perhaps, if the recrystallization is done near the freezing point of pyridine, unstable complexes may be obtained. Another indication in the same direction is the variations in the wave-length for the maximum in the visible region. This is 4350 Å in nonpolar solvents like carbon tetrachloride and benzene, but about 4600 Å in pyridine and 4500 Å in water.

Attempts to elucidate a bonding to particular groups in the protein molecule have not been successful. This is probably also not an easy task, as not even the nature of the copper-protein complex has been completely cleared up (cf. works of Macheboef and Viscontini, 1943, Fiess and Klotz, 1952, Tanford, 1952), and these bonds must be considerably weaker. An attempt was made to saturate the protein solution with Cu-DMDT, to see if after a certain amount was added, the rest could be recovered quantitatively, but this did not succeed. After addition of considerable amounts, the protein precipitated without seeming to have exhausted its binding capacity. Similar results have been obtained with cupric ions (Macheboef and Viscontini, 1943). The ratio of cupric ions to DMDT ions was found to be of only slight importance for the extractability. A little more was extracted in acid solution than in alkaline (something which may indicate an electrostatic interference cf. Fiess and Klotz, 1952) Between the different alkyl derivatives, no difference in extractability could be found. With peptone, the results were the same as with gelatin. After hydrolysis of gelatin with hydrochloric acid

and subsequent neutralization, the amino acid mixture obtained did not prevent the extraction. Thus the complex cannot be attributed to one single amino acid group, but rather to large peptide chains. Probably the bonding forces are so weak that bonds with monodentate ligands become very unstable. Bonds with bidentate ligands of sufficient rigidity are probably the only ones that have some stability, and they may only exist in solutions of large peptide chains.

That the bonds are weak is also shown by the observations of Linderholm and Berg (1951) that the addition of surface active compounds like ordinary soap and Tween 80 again changed the distribution equilibrium in favour of the non-polar phase.

C. Absorption Spectra of Dithiocarbamyl Compounds in Yeast Suspensions

When the method of Shibata, Benson and Calvin (1954) for determination of absorption spectra of suspensions of living microorganisms appeared, an effort was made to use it for a direct study of the state of the dithiocarbamyl compounds when added to intact yeast suspensions.

A yeast suspension containing one gram per liter of washed yeast in phosphate buffer of pH 6.3 was used for these studies. The heavy metal dithiocarbamates were added as mixtures of Na-DMDT and an equivalent amount of zinc or cupric sulfate. TMTD and TMTM were added as a solution in acetone to the dry and empty vessel, which was then evacuated by the water suction pump until all acetone had disappeared. The yeast suspension was then added and the flask shaken well. A set of suspensions or solutions of the dithiocarbamyl compounds in phosphate buffer without yeast was prepared simultaneously. The spectra were taken in the Cary recording spectrophotometer, with pieces of Whatman No. 1 filter paper soaked with mineral oil behind the cuvettes. The spectra of the yeast suspensions were taken against similar yeast suspensions without dithiocarbamyl compounds, and those of buffer systems against buffer solution. The spectra are seen in figure 14.

It is seen that when Na-DMDT has been added to the yeast suspension, lower absorption values are obtained than on the buffer solution, perhaps indicating a disappearance of the compound. The shape of the curve and the ratio between the two maxima has not been altered, indicating that no heavy metal complexes have been formed.

When Zn-DMDT has been added to the buffer solution, the spectrum indicates that a partial dissociation has taken place, and a mixture of Zn-DMDT (1:1) and free DMDT ions is obtained. In the yeast suspension, this dissociation has proceeded considerably further, so that Zn-DMDT (1:1) cannot be detected from the spectrum.

With Cu-DMDT, the absorption spectrum in phosphate buffer is very featureless, probably because of aggregation of the precipitated Cu-DMDT.

Table 4. Concentrations obtained in the supernatant in phosphate buffer incubated for one hour at 30° with various concentrations of dithiocarbamyl compounds, and centrifugated.

Start concentration, $\mu\text{M/l}$	Zn-DMDT	Cu-DMDT	TMTD	TMTM
100	60	4.7	62	97
50	28	4.2	50	51
20	12	1.8	18	21

In the yeast suspension, more details are seen in the spectrum, indicating a dispergating effect of the yeast cells on Cu-DMDT.

With TMTD, it is seen that addition of yeast has caused a considerable reduction of DMDT ions. The ratio between the maxima of curve 2 would suggest that about 70 per cent of the TMTD added has been reduced.

With TMTM, addition of yeast has caused no significant change in the absorption spectrum.

The results from this study corroborate those from the extraction tests reported earlier. But probably this method can give more reliable data about the actual chemical state of the dithiocarbamyl compounds when acting on yeast cells, since the extraction procedure may cause shifts in the equilibria.

V. Attempts to Determine the Uptake of Dithiocarbamyl Compounds by Yeast Suspensions

Miller, McCallan and Weed (1953) have investigated the uptake of labeled Fe-DMDT in various fungal spores (conidia) and in yeast cells. Their results seem to indicate — as with a number of other compounds tried — a very large accumulation of Fe-DMDT in the cells; up to 500–1000 ppm was reported. This must be far above the solubility of the compound. However, the manner in which their experiments were performed makes it difficult to conclude whether this amount virtually has been taken into the cells, whether it is an adsorption to the cell walls, or merely a coprecipitation.

At an early stage of the investigations, some attempts were made to determine the uptake of Na-DMDT, Zn-DMDT, Cu-DMDT, TMTD and TMTM by resting yeast cells, osmotically equilibrated against a phosphate buffer.

After having been shaken overnight, the yeast was twice washed by centrifugation and resuspension in phosphate buffer 0.1 *M*, pH 7.0. 200 grams (fresh weight) was suspended in 600 ml of the same buffer, to give a total volume of about 820 ml. This suspension was used for the experiments. The extra-cellular volume was determined by adding 2 ml caramel solution to 10 ml of the suspension, centrifuging, and determining the dilution of the caramel solution photometrically (at 4500 Å.). A correction was made for the extinction value obtained when adding 2 ml water to 10 ml of the suspension, centrifuging and measuring the solution in the photo-

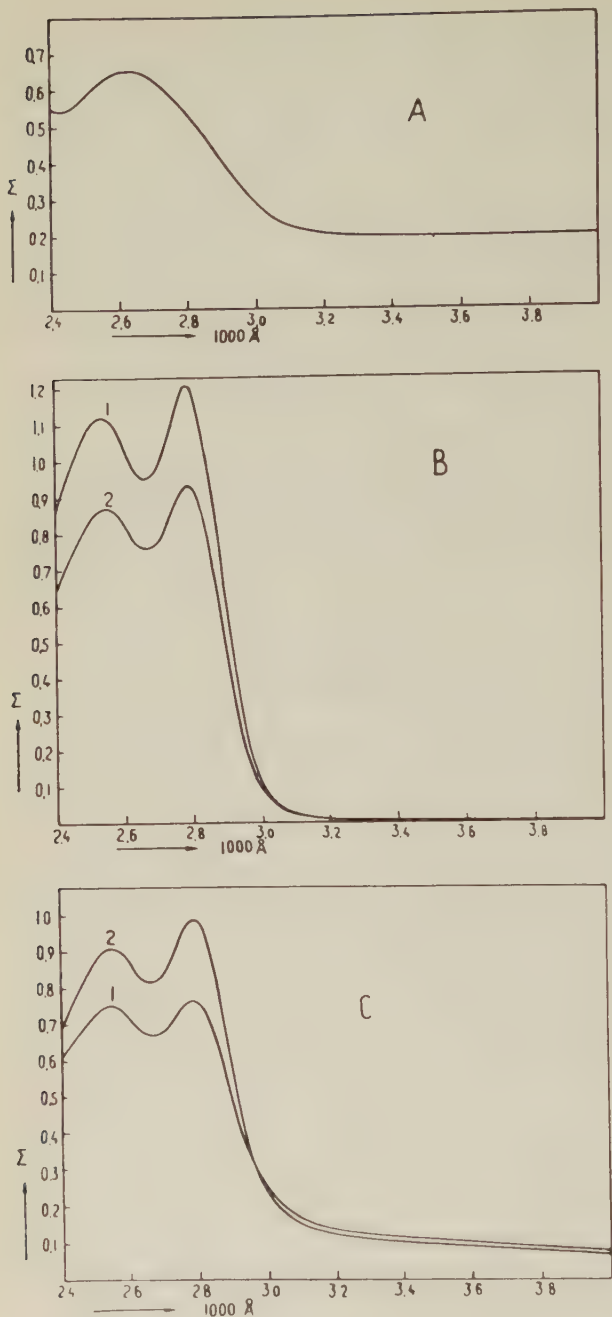


Figure 14, A, B, C.

A, Absorption spectrum of the yeast suspension (1 gram fresh weight per liter) against water.

B, curve 2, 100 μ M Na-DMDT in yeast suspension against yeast suspension without Na-DMDT. Curve 1, the same concentration of Na-DMDT in buffer against buffer alone.

C, curve 2, 100 μ M Na-DMDT and 50 μ M zinc sulfate in yeast suspension against yeast suspension. Curve 1, the same dithiocarbamate system in buffer against buffer alone.

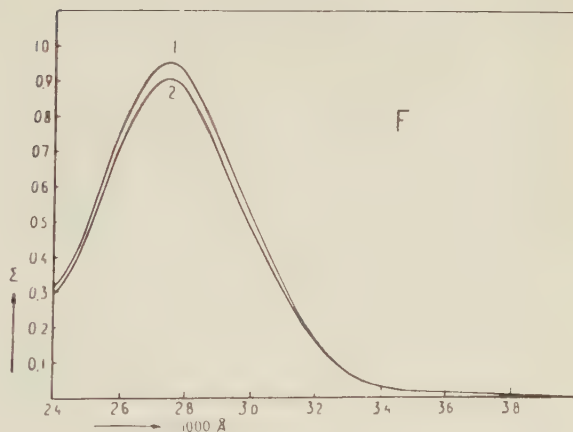
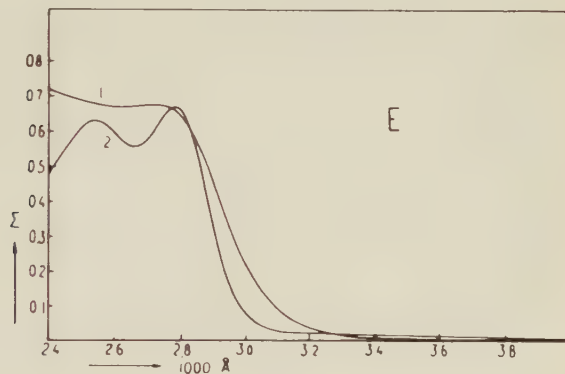
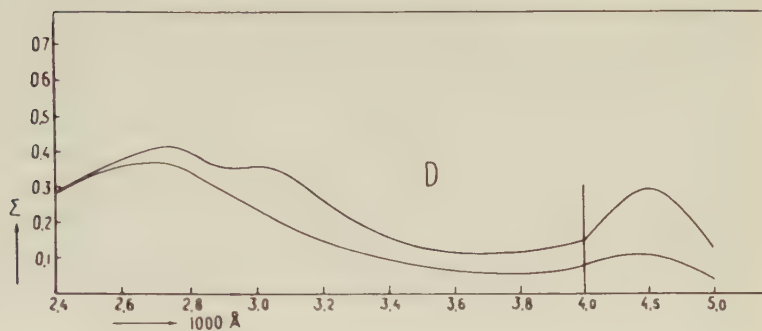


Figure 14, D, E, F.

D, as C, but with cupric sulfate added instead of zinc sulfate.

E, curve 2, 50 μ M TMTD in yeast suspension against yeast suspension, curve 1, 50 M TMTD in buffer against buffer.

F, as E, but with 50 μ M TMTM.

See the text for further details.

meter. For a discussion of this method, together with other methods for determination of the extra-cellular volume, see Malm (1949).

To 100 ml samples of the suspensions, agitated by magnetic stirring in the flasks, was added 10, 5 and 2 ml of 1-mM solutions or suspensions of the various dithiocarbamyl compounds. The concentration at the start was calculated on the basis of the total extra-cellular volume in each flask after addition of the dithiocarbamyl compound. After 2, 30 and 60 minutes 10 ml samples were withdrawn, centrifuged sharply for 5 minutes, and 5 ml of the clear supernatant extracted with the same volume tetra. The Na-DMDT series was extracted after the addition of a drop of 3 per cent cupric sulfate solution. To check whether the eventual disappearance of the compounds was due to an uptake (or surface adsorption), or merely to sedimentation of the amount exceeding the solubility, control flasks were prepared in the same manner as the yeast suspension flasks, but without yeast. From these flasks samples were withdrawn at the end of the experiment, centrifuged and extracted with tetra.

The results from the experiments are compiled in figure 15. In table 4 are seen the values obtained in the control flasks with phosphate buffer only. From comparing these results, one can see that for Cu-DMDT and to some extent for Zn-DMDT, it is not possible to state whether an uptake has occurred, or whether the compounds only have precipitated together with the yeast cells in the centrifuge. Na-DMDT has disappeared slowly after an initial rapid decrease in concentration. The disappearance may be due partly to accumulation in the cells, partly to decomposition. TMTD has disappeared far below what should be soluble. However, from the previous investigations TMTD is known to be reduced to DMDT ions, and this may also have occurred here. The curves for TMTM are rather peculiar. They seem to indicate a very rapid initial uptake, whereafter the values are surprisingly constant. It should be remembered that the hydrolysis of TMTM is a comparatively slow process.

The shape of the curve for TMTM is more similar to an extraction curve than to a permeation curve, since the equilibrium is obtained so rapidly (even if the total time of centrifugation is allowed for, complete equilibrium has been obtained in 7 minutes). Bearing in mind that the cells are surrounded by a membrane, the idea of a distribution of the compound between two phases would seem far from improbable. By analogy, it should then also be permissible to conclude that the same reaction will occur with TMTD, Zn-DMDT and Cu-DMDT.

Since the location of the absorption maximum of Cu-DMDT in the visible region is dependent on the polarity of the solution (page 753), an effort was made to find out whether Cu-DMDT was absorbed in lipides or still was in a polar phase, in a yeast suspension. For this experiment was used a yeast suspension containing 1 gram yeast per liter, and a Cu-DMDT concentration of 25 μ M, prepared in the yeast suspension by adding Na-DMDT and cupric

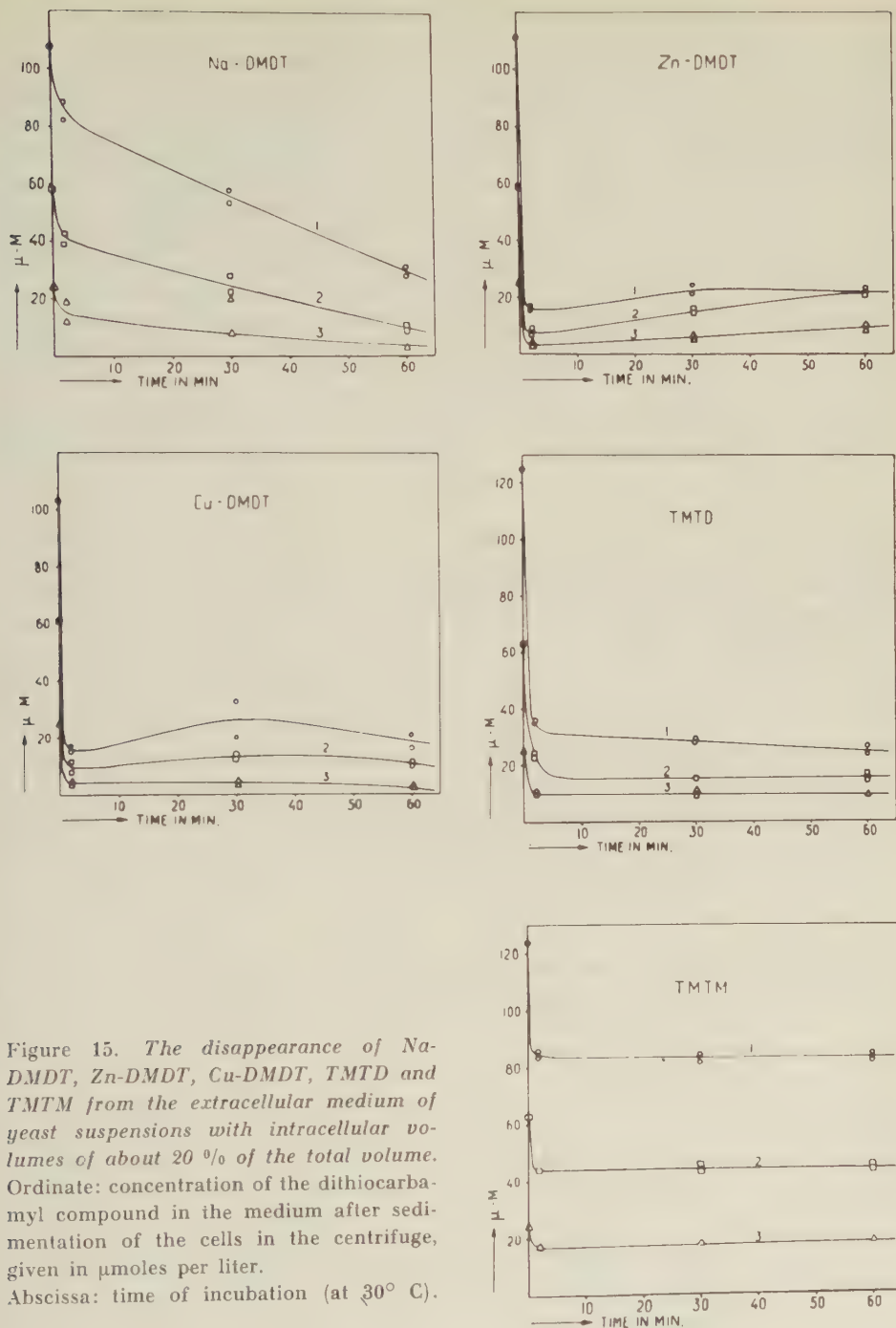


Figure 15. The disappearance of Na-DMDT, Zn-DMDT, Cu-DMDT, TMTD and TMTM from the extracellular medium of yeast suspensions with intracellular volumes of about 20 % of the total volume. Ordinate: concentration of the dithiocarbamyl compound in the medium after sedimentation of the cells in the centrifuge, given in $\mu\text{moles per liter}$. Abscissa: time of incubation (at 30°C).

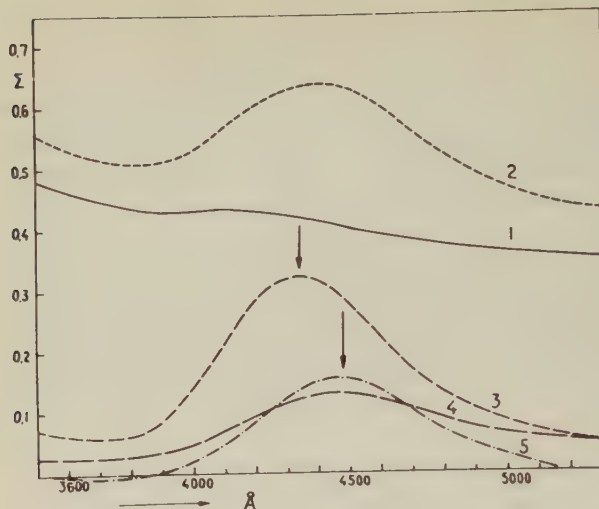


Figure 16. Location of the maximum of Cu-DMDT in the visible region. Curve 1, spectrum of the yeast suspension (1 g/liter) against water. Curve 2, 25 μ M Cu-DMDT added to the yeast suspension, against water. Curve 3, 25 μ M Cu-DMDT in tetra. Curve 4, 25 μ M Cu-DMDT in water. Curve 5, 25 μ M Cu-DMDT in yeast suspension against the same yeast suspension alone. See the text for technical details.

sulfate in the proper concentrations. The spectrophotometric technique developed by Shibata, Benson and Calvin (1954) was used, and the results are shown in Figure 16.

It is seen that in the yeast suspension, the absorption maximum is lying closely as it does in water. Under the conditions in this experiment, the cupric atom in the Cu-DMDT complex is thus still surrounded by polar molecules to a large degree. This does not necessarily rule out the possibility of an *adsorption* of the complex at a nonpolar phase-border by the lipophilic dimethyl groups in either end of the molecule, but the most natural conclusion would probably be that a possibly occurring ad-(or ab)-sorption is to a polar phase-border.

VI. The Effect of Dithiocarbamyl Compounds on the Oxidative Reactions in Intact and Resting Yeast Cells

A. Methods

Baker's yeast, washed by suspending in distilled water, centrifuging and re-suspending, was used for all investigations. The substrate consisted of the various carbon sources in 0.02 *M* potassium phosphate buffer of pH 6.2–6.3. Experimental temperature was 30 °C throughout the work. A circular Warburg apparatus with 14 flasks (B. Braun, Melsungen) of approximately 15 ml volume, and of the ordinary shape with one side arm and gas vent through the side arm stopper was used for determination of the gas exchanges. The flask constants were determined by a method not previously seen described in the literature. It is mentioned here, owing to its rapidity and simplicity.

The flasks, connected with the manometers, were attached to a burette by means of the side arm stopper and a short rubber tubing. The burette was filled to a known position with alcohol, the alcohol also filling the rubber tubing and the bore in the side arm stopper. By opening the burette stopcock, the Warburg flask was filled with alcohol. To avoid trapping of air, the manometer holding the flask was carefully tilted, and alcohol let in until it reached a point on the horizontal arm of the manometer. This was marked, and the volume of the flask and manometer to this point simply read on the burette. The volume in the rest of the manometer was determined in one of the ordinary ways. The method can be used on all flasks having a side arm vent, and on most shapes of flask. Its advantage is its rapidity, and the fact that replicate determinations are easily made. The accuracy (about ± 0.05 ml) is sufficient for most manometric work.

The Warburg flasks were normally filled with 2 ml of yeast suspension, and the side arm with 0.2 ml of the solution of the substance to be added during the experiment. The yeast suspension contained (when nothing else is stated) 1 gram fresh weight of yeast per liter.

The gas exchanges are in all experiments reported as the volumes actually determined in each experiment, as it was not felt to be of importance in this work to operate with the *Q* values.

Oxidation of Na-DMDT. As Na-DMDT is oxidized in air, it was necessary before starting the manometric study to check whether the concentration of Na-DMDT used would cause an oxygen uptake that could introduce errors into the experiments. The oxidation of one μ -mole Na-DMDT to TMTD by atmospheric oxygen will cause an oxygen uptake of 11.2 μ l. Normally, the Warburg flasks contain 2 ml yeast suspension with a maximal Na-DMDT concentration of 200 μ moles per liter. Thus, the maximal oxygen uptake would be 4.4 μ l. Even if the oxidation is complete, the error introduced will be small, when gas exchanges above 100 μ l are measured. To check these theoretical considerations, the pressure decrease in a flask containing 0.5 μ moles of Na-DMDT in phosphate buffer was observed. After 5 hours at 30°C, the pressure decrease corresponded to an oxygen uptake of 3.0 μ l.

James and Garton (1952) studied the pressure changes shown by Na-DEDT. They found a gas *evolution* of maximum 20 μ l by 2.5 ml 1000 μ M Na-DEDT. This was by them ascribed to COS evolution, but it is more likely to be caused by the formation of carbon disulfide. Even if such a gas evolution should occur, it could at most be 2 μ l with the concentrations used.

The Endogenous Respiration. In the investigations reported here, oxygen uptake has been in each case corrected for endogenous respiration. It may be doubtful if the endogenous oxygen uptake (which must be due to the respiration of reserve carbohydrates, fats, amino acids and other products in the cells) proceeds at the same rate in the presence of large excesses of easily oxidizable substrates, but the fact that the endogenous respiration seems unaffected by the dithiocarbamyl compounds (see table 7) makes it justifiable to correct when strong inhibition of the oxidation of the added substrate has occurred. It seems then most logical to correct in all cases.

B. Introductory Experiments with Glucose as the C-Source

As already mentioned, experiments reported in the literature have shown that the dithiocarbamyl compounds affect only the oxygen uptake of fungi

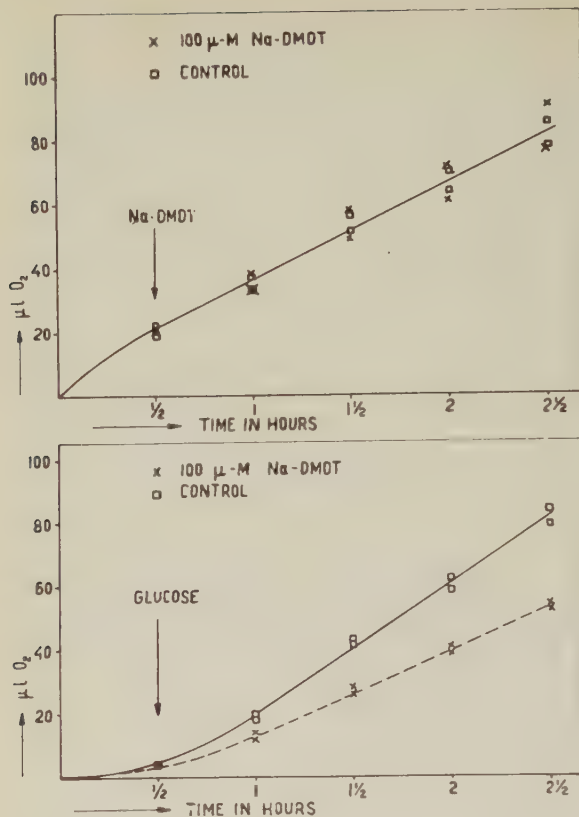


Figure 17. The oxygen uptake of yeast suspensions with glucose as the C-source, and with Na-DMDT. Upper curves, glucose (0.1 M) added from the start, Na-DMDT (to a final concentration of 100 μ M) added after $1/2$ hour. Lower curves, Na-DMDT (100 μ M) added from the start, glucose (to a final concentration of 0.1 M) added after $1/2$ hour.

in concentrations considerably above those suppressing the growth completely (Klöpping, 1951). Our experiments verified this. The respiration of yeast suspensions in phosphate with glucose as the C-source was not significantly inhibited by concentrations of Na-DMDT below 100 μ M, and if Na-DMDT was added after the glucose.

A detectable inhibition was, however, obtained when glucose was added to a similar yeast suspension incubated with Na-DMDT for a short time ($1/2$ –1 hour). The curves from an experiment showing this are seen in figure 17.

Various pathways have been proposed for the reaction sequence leading to the complete oxidation of glucose to carbon dioxide and water in yeast cells. Whereas the glycolytic route to pyruvic acid for some time has been assumed to be the sole passage through the anaerobic stages, new investigations stress the importance of the so-called Dickens scheme or oxidative shunt, which by passing through phosphogluconic acid, ribulose-5-phosphate

and glyceraldehyde-3-phosphate, leads to pyruvic acid. (Bloom and De Witt Stetten Jr., 1953, Beevers and Gibbs, 1954, Blumenthal, Lewis and Weinhouse, 1954). The latter authors estimated that under aerobic conditions the extent of the oxidative shunt ranged from zero to 30 per cent in *Saccharomyces cerevisiae*, and in *Torula utilis* from 30 to 50 per cent. Under anaerobic conditions, at least 95 per cent of the glucose was catabolized via the Emden-Meyerhof pathway.

From pyruvic acid on, there is not complete agreement regarding the relative importance of the condensing enzyme system leading through the tricarboxylic acids to succinic acid, compared with the direct formation of succinic acid from acetyl coenzyme A (the dicarboxylic or Thunberg-Wieland cycle) as respiratory pathways. It has by several, particularly earlier, workers been felt that the main purpose for the operation of the Krebs cycle in yeast was to produce metabolites needed in cellular synthesis (cf. Krebs, 1943). However, as better methods for obtaining cell-free preparations of yeast have been developed, and evidence in tracer experiments is collected, the importance of the tricarboxylic cycle as ordinary oxidative route has been reconsidered (Guzman Barron and Ghiretti, 1953, Nossal, 1954 a and c).

Otherwise, the knowledge of the breakdown pathways of carbohydrates in fungi is very scanty. Evidence for the operation of a condensing enzyme system forming citric acid from acetyl coenzyme A and oxalacetic acid in *Aspergillus niger* was obtained by Ramakrishnan and Martin (1954 a and b). Earlier, Urba and Burger (1952) were not able to find a correlation between coenzyme A activity and the formation of citric acid. In *Blastocladiella emersonii*, Cantino and Hyatt (1953) have demonstrated the presence of the tricarboxylic acid cycle.

C. The Oxidation of Acetate. The Effect of Heavy Metals as Synergists for Na-DMDT

One of the enzymatic sequences in the oxidation of glucose consists of the oxidative decarboxylation of pyruvic acid, with the formation of acetyl coenzyme A. Following the formation of this compound, a transacetylation takes place, either mediated by the condensing enzyme and leading to the formation of citric acid, or by another not yet completely understood mechanism, leading to the formation of succinic acid (or succinyl coenzyme A). In order to study the direct combination of acetate with coenzyme A, together with the cyclophorase part of the respiratory scheme, some experiments were done with acetate as the C-source. (Evidence for the oxidation of acetate in yeast via acetyl coenzyme A and the tricarboxylic route has been furnished by Guzman Barron and Ghiretti (1953) and by Nossal

(1954 c)). Further, Novelli and Lipmann (1947) have demonstrated the importance of coenzyme A for the oxidation of acetate in yeast. Acetyl coenzyme A was also isolated for the first time from yeast (Lynen, Reichert and Rueff, 1951).

It was found that the yeast used for these experiments oxidized acetate rapidly, and with a brief lag period. It has been noted by Eaton and Klein (1954) that *Saccaromyces cerevisiae* cannot oxidize acetate in the early logarithmic phase, although the oxidation in the late logarithmic and the stationary phase may be as rapid or more so, as with glucose as the C-source. The acetate *assimilation*, measured by tracer technique, was found to proceed rapidly even in the early growth phases. Similarly, Vignais and Vignais (1954) found only a slight acetate oxidation by *Escherichia coli* in the early logarithmic phase. At the end of the first third of the logarithmic phase they found lag periods of 30 minutes, and at the end of the logarithmic phase a rapid oxidation without lag. The yeast used in the respiration experiments may in accordance with these results be stated physiologically to be in the stationary state of growth. This is quite natural, since the factory producing the yeast certainly makes use of the full period of logarithmic growth, to obtain as high a yield as possible.

The results from the respiratory experiments showed that *the acetate oxidation was considerably more sensitive towards Na-DMDT than was the glucose oxidation*.

This led to further studies of the effect of Na-DMDT on the oxidation of acetate. By chance it was observed that the inhibition was strongly increased when a complete mineral salt solution (solution 1, p. 738) was added to the phosphate buffer. In this case, marked inhibition occurred at concentrations as low as those that caused growth inhibition.

To find out which of the components in the salt solution was responsible for the synergistic effects, a screening experiment was made, in which the various compounds were added to the yeast suspensions in the Warburg flasks, Na-DMDT being tipped in from the side arm. Figure 18 shows that zinc plays a dominant role as synergist, followed by iron, and the trace elements in the tap water (probably iron). The effects of zinc and copper were then studied more closely. (Figures 19 and 20).

As the addition of zinc sulfate or cupric sulfate to a phosphate buffer probably makes it very unbalanced, and depresses the metabolic rates, a study was made of how the respiration of acetate was affected by various concentrations of these salts. Figure 21 shows that both salts, but particularly cupric sulfate, inhibit the respiration. The curves in figures 19 and 20 should consequently be somewhat corrected to give a true picture of the effect of

Figure 18. The oxygen uptake in yeast suspensions with 0.1 M sodium acetate as the C source. Curves marked b: Na-DMDT (100 μ M/l) added after 1 hour. Curves marked a are control curves without Na-DMDT. Phosphate buffer, to which has been added: Curves 1, 40 % of the mineral salt solution. Curves 2, 500 mg KCl per liter. Curves 3, 10 mg FeSO_4 per liter. Curves 4, 40 % tap water. Curves 5, 10 mg $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ per liter. Curves 6, 10 mg ZnSO_4 per liter.

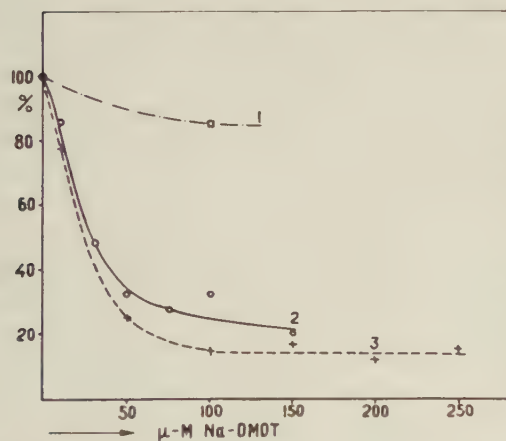
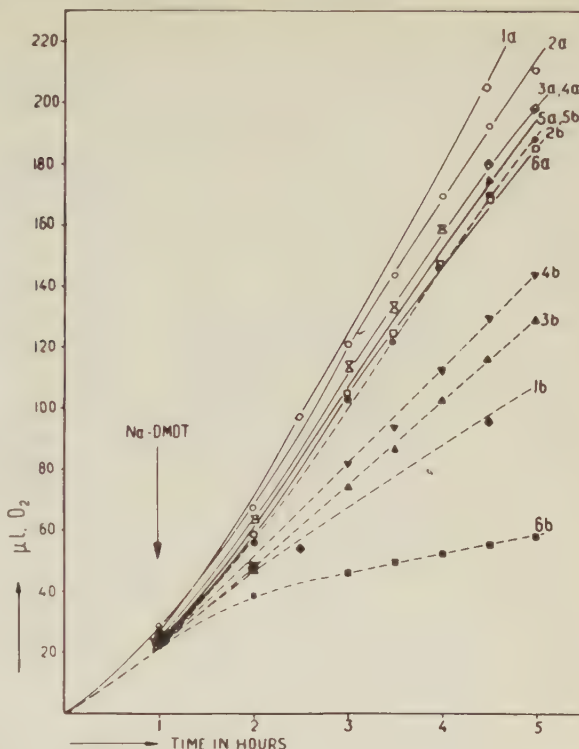


Figure 19. The oxygen uptake in yeast suspensions with 0.1 M sodium acetate as the C-source, and with varying concentrations of Na-DMDT and zinc sulfate. Curve 1, no zinc sulfate. Curve 2, 25 μ M, and curve 3, 50 μ M zinc sulfate. Ordinate: the oxygen uptake in the period 0—4 hours after the addition of Na-DMDT in per cent of that without Na-DMDT (but with the same zinc sulfate concentration)

the dithiocarbamate complexes alone. Thus, the curves in figure 20 would probably approach the value 100 per cent at higher concentrations of Na-DMDT.

Turning first to the curves in figure 19, where zinc sulfate has been added as the single heavy metal salt, it is seen that the inhibitory effect is dependent

Figure 20. The oxygen uptake in yeast suspensions with sodium acetate as the C-source, and with varying concentrations of Na-DMDT and cupric sulfate. Curve 1, 50 μ M, curves 2 and 3, 25 μ M cupric sulfate. Ordinate: as in fig. 19.

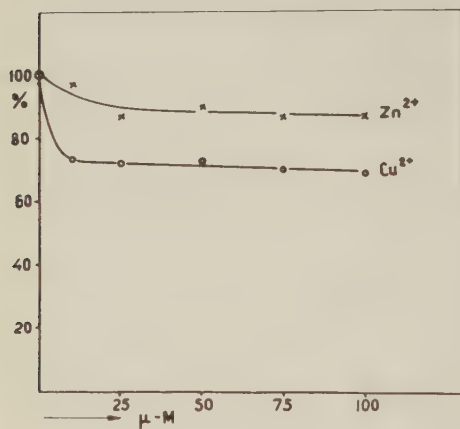
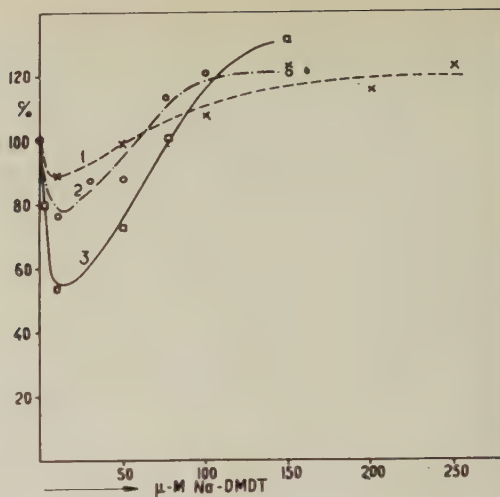


Figure 21. The oxygen uptake of yeast suspensions with 0.1 M sodium acetate as the C-source, at various concentrations of zinc sulfate and cupric sulfate. Ordinate: oxygen uptake in the period 0—4 hours in per cent of that without added heavy metals. Abscissa: concentrations of the respective heavy metal salts in μ moles per liter.

on the amount of Zn-DMDT formed. But it cannot be stated whether the effect is dependent on the 1 : 1 or the 1 : 2 complex, or on both. Seemingly, Na-DMDT or the DMDT ion itself has only a slight effect. This can be explained by the fact that the DMDT ion is unstable, and it may be hydrolyzed before having time to act, if no chelating heavy metals are present. To check this, an experiment was done in which zinc sulfate was added from the side arm after 2 hours, the main compartment of the flask containing yeast suspension with Na acetate and 100 μ M Na-DMDT. The curves obtained are seen in figure 22. They show that for the most part, Na-DMDT must have been rendered unavailable during the time of incubation. There is, however, a definite increase in inhibition following the addition of zinc

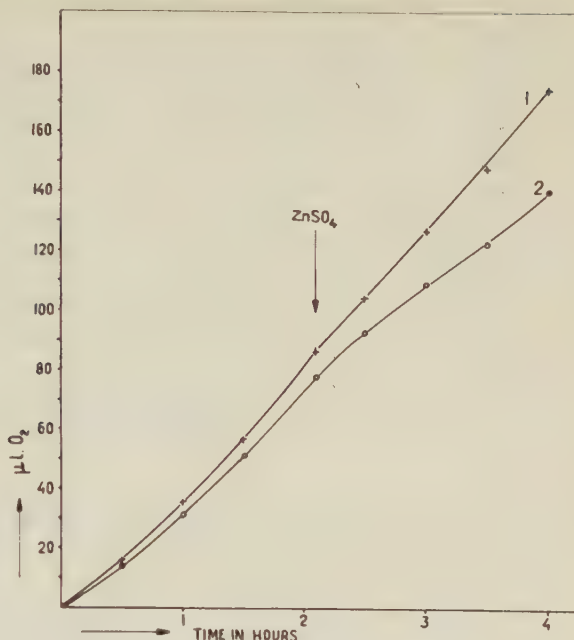


Figure 22. The oxygen uptake of yeast suspensions with 0.1 M sodium acetate as the C-source. At start, 100 μM Na-DMDT was added in one series (curve 2). After 2 hours, zinc sulfate to a final concentration of 50 μM per liter was added in both series.

sulfate, demonstrating that even if the instability of Na-DMDT is accounted for, Zn-DMDT is quite a lot more toxic than Na-DMDT.

We shall now turn to the curves in figure 20, which gives the results from the experiment with cupric sulfate as the sole heavy metal added. The rather peculiar shape of these curves should be considered in view of the discussion in Chapter IV A. It will be remembered that it was found that the formation of the ordinary 1 : 2 complex of Cu-DMDT probably must pass over the 1 : 1 complex, which, under the conditions used in the experiments reported on p. 745 (figure 8) had a maximal concentration when 5—8 μmoles Na-DMDT per liter were added. From this comparison, the conclusion must be drawn that Cu-DMDT (1 : 1) exerts a very inhibitory action on the oxidation of acetate, while Cu-DMDT (1 : 2) must be absolutely non-toxic, perhaps because of its low solubility.

D. The Inversion Phenomenon

In a mixture of zinc and cupric salts the formation of Zn-DMDT complexes can only take place when all cupric ions have been bound as Cu-DMDT, owing to the greater stability constants of the latter compound. From this can be concluded that in a mixture of these salts, the effect of Na-DMDT on acetate oxidation will be dependent on the Na-DMDT-con-

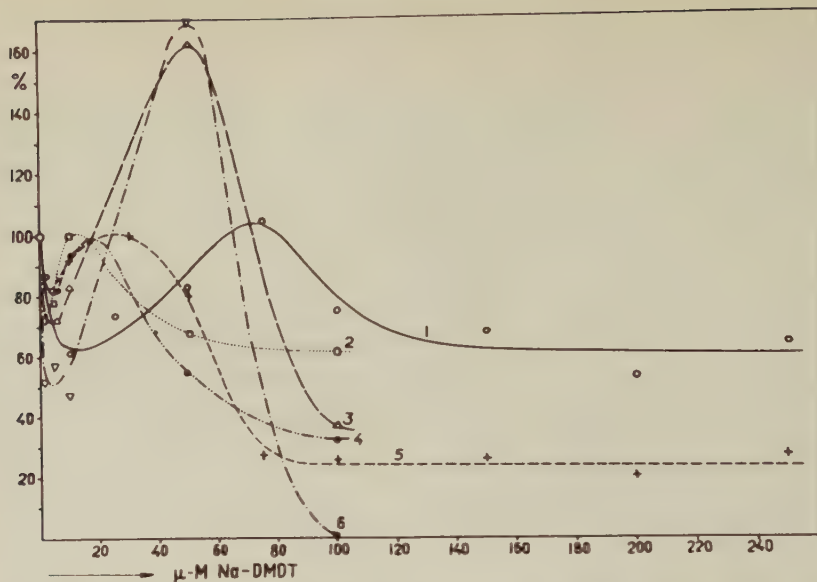


Figure 23. The oxygen uptake of yeast suspensions with sodium acetate as the C-source, as a function of the concentration of Na-DMDT, and under different conditions. Curve 1, 1 gram yeast per liter, 20 μ M zinc and 20 μ M cupric sulfate. Curve 2, 200 mg yeast per liter, 20 % mineral salt solution. Curve 3, 20 μ M zinc and 20 μ M cupric sulfate per liter, 2 grams yeast per liter. Curve 4, 2 grams yeast per liter, 20 % mineral salt solution. Curve 5, 1 gram yeast per liter, 20 % mineral salt solution. Curve 6, 200 mg yeast per liter, 20 μ M zinc and 20 μ M cupric sulfate per liter. Ordinate: oxygen uptake during 4 hours in per cent of that without Na-DMDT.

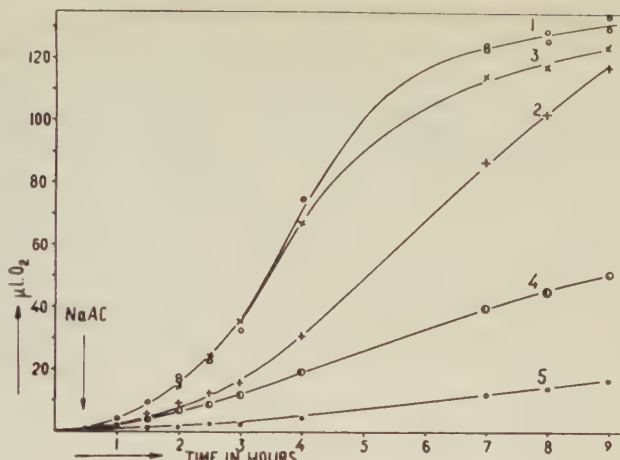
centration in a manner giving a typical inversion phenomenon, and the area corresponding to no effect will lie at the concentration of Na-DMDT, which is equivalent to the cupric ions for the formation of Cu-DMDT (1:2).

A number of experiments verified this. Some of the curves are shown on fig. 23.

Thus, it has been shown that a phenomenon, corresponding in effect very closely to the inversion phenomenon reported by a number of authors on the growth of microorganisms in the presence of dithiocarbamyl compounds, can be demonstrated on the acetate oxidation of yeast by Na-DMDT. It is dependent on the formation of the following heavy metal complexes: Cu-DMDT (1:1), causing the inhibition at lower concentrations of Na-DMDT, Cu-DMDT (1:2), causing reversal of the inhibition, and Zn-DMDT complexes, causing new inhibition.

By chemical analogy, it may be concluded that Fe-DMDT and Mn-DMDT will act similarly to Zn-DMDT in this scheme.

Figure 24. *The oxidation of acetate by yeast.* Start concentration of acetate, 0.001 *M* (2 μ moles in each flask). Phosphate buffer with mineral salt solution added. Curve 1, control without Na-DMDT. Curves 2—4, with 1, 10, 100 μ *M* Na-DMDT added, respectively. Curve 5, endogenous respiration (without acetate).



Many of the seemingly controversial results reported in the literature regarding, e.g. on one hand the antagonistic action of cupric salts to the toxicity of Na-DMDT, and on the other, the fungitoxicity of Cu-DMDT, can be explained in the light of these findings.

The results of Raalte (1952) are interesting in this case, since from them it seems that he was near a discovery of the connection between the cupric ions and the inversion phenomenon (see his tables 2 and 3). A brief communication of these findings has previously been published (Goksøyr, 1955).

Of course great care must be taken before the results reported here are taken as valid explanation for the growth phenomenon. For instance, there can be little doubt that at concentrations of Na-DMDT above the inversion point, a lack of copper may be as limiting for the growth as is the effect of Zn-DMDT.

The effect of the concentration of the yeast suspension on the degree of inhibition was studied in some experiments. The curves obtained are also shown on figure 23. They are not very decisive. It is generally noted that the degree of inhibition of the Cu-DMDT (1:1) is extremely variable in all experiments. The reason for this is not quite clear. Part of the reason may be that the yeast cells carry varying amounts of heavy metals with them, probably absorbed on the surface.

The degree of inhibition was found to be practically independent of the acetate concentration. It did not differ significantly with acetate concentrations of 0.1, 0.01 and 0.001 *M*. Figure 24 also shows clearly that even when the available acetate decreases to zero, the degree of inhibition does not increase. In fact, a stimulation seems to occur. Even if, because of the long time which the experiment lasted, the dithiocarbamate concentration should

Table 5. *The effect of incubation with calcium pantothenate on the inhibition of acetate oxidation by dithiocarbamate in yeast. See the text for details.*

Flask No.	Pantoth. treated	Acetate added	Na-DMDT added	O ₂ consumption	Corrected for endogenous	% inhibition
1	+	No.	No.	87.5		
2	+	4 μ -moles	"	161.5	74.0	
3	+	"	"	151.5	64.0	
4	+	"	"	158.0	70.5	
5	+	"	2 μ -M	108.0	20.5	} 60.5
6	+	"	"	115.0	27.5	
7	—	No.	No.	78.0		
8	—	4 μ -moles	"	151.5	73.5	
9	—	"	"	155.5	77.5	
10	—	"	"	146.0	68.0	
11	—	"	2 μ -M	88.0	10.0	} 84
12	—	"	"	91.0	13.0	

be considerably lower at the end of the experiment than at the start, it would seem quite clear that the inhibition cannot be due to a competition between acetate and dithiocarbamate.

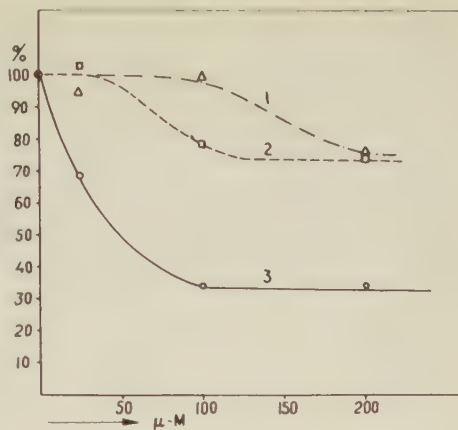
Novelli and Lipmann (1947) found that incubation with pantothenic acid, resulting in an increase in the coenzyme A content, led to an increased ability of yeast to oxidize acetate.

An attempt was made to find out whether a similar treatment resulted in a reduced degree of inhibition of the oxidation of acetate by dithiocarbamates.

A yeast suspension, containing 2 grams fresh weight per liter in a phosphate buffer of pH 6.2 and with 0.5 per cent glucose added, was shaken overnight at room temperature, one portion with 50 mg calcium pantothenate per liter added, another without. The next day the yeast was washed, transferred to new buffer solutions with 20 μ M cupric sulfate and 50 μ M zinc sulfate added. 2 ml samples of the suspensions were transferred to Warburg flasks, and 0.2 ml 20 μ M Na-DMDT added. In the side arms were placed 0.2 ml 0.02 μ M sodium acetate. The flasks were tipped one hour after the readings had started.

The results are seen in table 5. They show that the respiratory rate has not been increased, but it seems as if a significant reduction of the inhibition has occurred after the treatment with calcium pantothenate. This might permit the conclusion that increase in the coenzyme A content results in a lower sensitivity of the acetate oxidation mechanism in yeast towards the dithiocarbamyl compounds.

Figure 25. The oxygen uptake of yeast suspensions with 0.1 M sodium acetate as the C-source, at various concentrations of Na-DPDT (curve 1), Na-DEDT (curve 2) and Na-DMDT (curve 3). To the substrate was added 20 μ M zinc sulfate. Ordinate: oxygen uptake, in per cent of that without dithiocarbamates during 0–4 hours.



E. The Effect of Other Dithiocarbamyl Compounds on the Acetate Oxidation

Since it now has been possible to demonstrate an inhibition by Na-DMDT on a particular metabolic scheme at concentrations whose order of magnitude corresponds to those causing growth inhibition, and with a similar pattern of action, it was thought worth while to use the acetate oxidation as a model system, and also to study the effect of Na-DEDT, Na-DPDT, TMTD and TMTM on this system.

A screening experiment showed that for all, the inhibition increased when a complete salt solution was added to the substrate.

In the presence of 20 μ M zinc sulfate, the inhibition of dithiocarbamates decreased in the order methyl > ethyl > propyl. This can be seen from figure 25.

With TMTD, it would be of particular interest to see if heavy metal salts had any action, since this would furnish evidence as to whether TMTD acts as such or only in reduced form. Experiments were therefore done to compare the effect on the acetate oxidation in the absence of heavy metal salts, and in the presence of 50 μ M zinc sulfate and 50 μ M cupric sulfate. Figure 26 shows that TMTD in itself must have an inhibitory action, as the inhibition increases continually with increasing concentrations above 20 μ M TMTD. An inversion effect is seen at lower concentrations, with the first inhibition maximum around 1–2 μ M TMTD. However, when zinc has been added, the inversion effect has disappeared, and the inhibition is considerably increased. With cupric ions present, the inversion effect is somewhat more pronounced, and a seemingly stimulatory effect seen around 20 μ M TMTD. From there on, the curve follows closely that of TMTD alone.

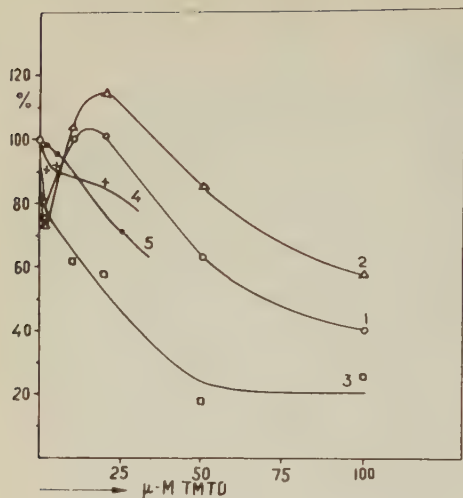


Figure 26. *The oxygen uptake of yeast suspensions with 0.1 M sodium acetate as the C-source, at various concentrations of TMTD. Ordinate: oxygen uptake during 0–4 hours in per cent of controls without TMTD. Curve 1, no heavy metals added. Curve 2, 50 μ M cupric sulfate added. Curve 3, 50 μ M zinc sulfate added. Curve 4, no heavy metals added. Curve 5, 50 μ M Na-DMDT, no heavy metals added.*

These findings can best be explained by assuming that TMTD is totally reduced at lower concentrations, and partly reduced at concentrations above 20–30 μ M. At the lower concentrations, traces of cupric ions have probably caused the inversion phenomenon. In some cases such traces were found to come from the acetone used as solvent for TMTD. To check whether the inversion effect really was due to the formation of Cu-DMDT (1:1), the experiment was repeated with TMTD dissolved in tetra. To one series was added 50 μ M Na-DMDT. This would transfer all copper present to inactive Cu-DMDT (1:2). Curves 4 and 5 in figure 26 show that with the purified system there are still traces of inversion effect to be seen, but the addition of Na-DMDT has led to a complete disappearance of the effect. This seems to prove that the inversion effect with TMTD is due to reduction of TMTD and the formation, successively, of Cu-DMDT (1:1) and Cu-DMDT (1:2).

The technical difficulty of adding the sparingly soluble TMTD to the yeast suspension in the Warburg flasks was overcome in the following manner: to the dry and empty flasks were added the desired amounts of TMTD dissolved in acetone (or tetra). The solvent was evaporated by placing the flasks in a drying cabinet at 60° C for a few minutes. Thereafter the yeast suspension to which acetate and, in some cases, heavy metal salts had already been added, was pipetted in to the flasks.

The effect of TMTM is seen from figure 27. The curves show that TMTM in itself is not inhibitory to the oxidation of acetate at concentrations below 200 μ M, but that its decomposition products in combination with zinc ions are inhibitory, and are even more so in combination with cupric ions. This indicates a hydrolysis, resulting in the formation of small amounts of DMDT ions which may combine with zinc or cupric ions.

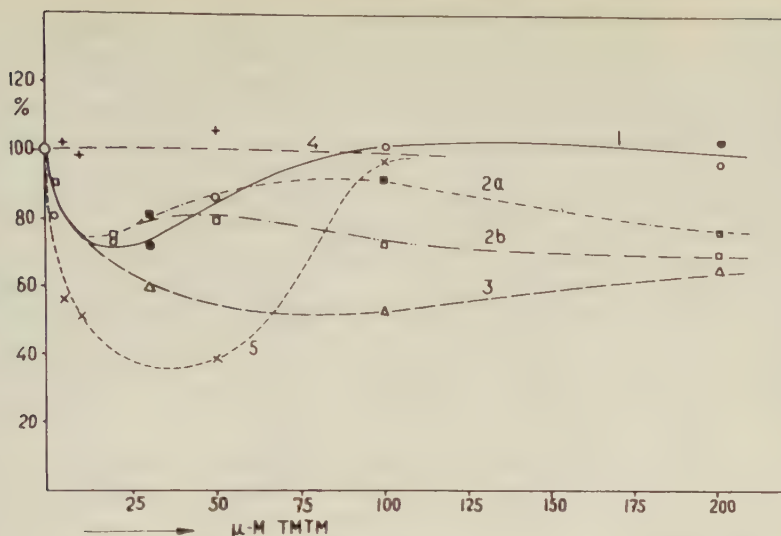


Figure 27. The oxygen uptake of yeast suspensions with 0.1 M sodium acetate as the C-source, at various concentrations of TMTM. Ordinate: oxygen uptake in per cent of that without TMTM, in the period 0—4 hours. Curve No. 1, no heavy metals added. Curve No. 2, 50 μ M zinc sulfate added (a and b denote two different experiments). Curve No. 3, 50 μ M cupric sulfate added. Curve No. 4, 50 μ M Na-DMDT, no heavy metals added. Curve No. 5, no heavy metals added.

An experiment was also done to compare the inversion effect in the presence of TMTM and 50 μ M Na-DMDT with that in the presence of TMTM alone. Curves 4 and 5 in figure 27 show clearly that Na-DMDT counteracts the inversion, which must thus be due to Cu-DMDT (1 : 1). It is worth while to note that the inversion part of curves 1 and 2 is drawn out along the abscissa, compared with Na-DMDT and TMTD. This is in agreement with the assumption of a slow hydrolysis of TMTM, leaving only a fraction of the compound active at a time.

F. The Effect of Histidine and Chelating Agents on the Na-DMDT Action

Sijpesteijn and van der Kerk (1951) found that L-histidine counteracted the effect of dithiocarbamyl compounds. Since in the previous experiments the role of heavy metals in the action of the dithiocarbamyl compounds has been revealed, attention was now turned towards a possible mechanism by which histidine might act.

The value of $\log k_1 k_2$ for the cupric complex of histidine is given (Martell

Table 6. *The effects of D,L-histidine and Versene on the inhibition of acetate oxidation by yeast suspensions, caused by Na-DMDT and heavy metal salts.* The experiments with histidine have been carried out in phosphate buffer to which was added 20 μ M cupric sulfate and 40 μ M zinc sulfate. The experiments with Versene were carried out in a phosphate buffer with 50 μ M zinc sulfate alone. — The figures given in the table show the oxygen consumption during the period 0—4 hours after addition of Na-DMDT from the side arm to the yeast suspension in the main compartment of the flask.

Na-DMDT conc. μ M	Exp. 1		Exp. 2		Exp. 3	
	μ l O ₂ no hist.	μ l O ₂ 1 mM hist.	μ l O ₂ no hist.	μ l O ₂ 25 mM hist.	μ l O ₂ no Vers.	μ l O ₂ 5 mM Vers.
0	55.0	102.0	64.5	100.5	122.5	96.5 102.5
2	45.5	94.5	45.0	77.0		
5	24.5	43.0	43.0	57.0		
10	10.5	35.5	73.0	42.0		
50	60.0	58.5	76.0	67.5		
100	26.0	44.0	9.0	36.0	20.0	86.0 92.0

and Calvin, 1952) as 18.33, and for the zinc complex as 12.88. These values are probably higher than the corresponding values for Cu-DMDT and Zn-DMDT (for the last complex a rough approximation gave a value around 9). Thus, in the presence of excess histidine, and at higher pH values, (the concentrations used by Sijpesteijn and van der Kerk were 0.04 per cent and more; that is approximately 2.5 mM histidine, or one thousand times the concentration of the fungicide), the heavy metals may mainly combine with histidine, and thus inactivate the dithiocarbamyl compounds.

Experiments to see whether this explanation would hold true were, accordingly, carried out, with Na acetate oxidation as the model system. In these experiments, DL-histidine was used. Additional experiments were also carried out with Versene (ethylene-diamine-tetraacetic acid) as another chelating agent. The results are compiled in table 6. The effect of Versene as competitor for zinc ions is very obvious, but the effect of histidine is not so clear. A distinct action has occurred, but it is very far from eliminating the total effect of the heavy metals added. It should be noted that Sijpesteijn and van der Kerk only found an effect of histidine on the first zone of growth inhibition, and absolutely no effect on the second zone. The effect found on the acetate oxidation seems to result in a flattening of the inversion area, and a similar effect might well in a qualitative growth experiment be interpreted as a counteraction of the first zone of inhibition. These considerations would also give a very natural answer to the findings later published by Sijpesteijn and van der Kerk (1954b), that a variety of

Table 7. *The effect of Zn-DMDT on different oxidative processes in intact yeast cells.* Standard procedure: 1 gram fresh yeast suspended per liter in phosphate buffer of pH 6.3, to which was added 0.1 M of the carbon source and 50 μ M zinc sulfate. Enough Na-DMDT to give a final concentration of 100 μ M was tipped in from the side arm at the start of the experiment. The values for uptake of oxygen or carbon dioxide evolution given in the table are those measured in the period 0—4 hours after the addition of Na-DMDT. For calculation of the inhibition, the values are corrected for endogenous respiration, determined by blank experiments without C-source.

C.-source	Without Na-DMDT	Endogenous	With Na-DMDT	Endogenous	% inhibition
Glucose, aerobic.....	119.5	14.5	73.0	15.5	45
Glucose, anaerobic... (CO ₂ evolution)	210.0		97.0		54
Ethanol.....	134.5	14.5	67.0	15.5	57
Succinic acid.....	(20.0)	12.5	28.0	14.0	15
	28.0	12.5	27.0	14.5	
	30.5	14.0	28.0	15.0	20
	28.5	24.0	32.5	29.5	
Butyric acid.....	39.5	24.0	42.0	29.5	10
	15.5	5.5	18.0	7.0	0
Tyrosine.....	24.5	20.0	33.0	22.0	—
	31.5	20.0	33.0	22.0	0
	26.5	12.5	34.5	14.5	—
Acetic acid.....	105.0	14.5	29.0	15.5	84

imidazole compounds are able to counteract Na-DMDT. All the compounds found to be effective are powerful chelating agents.

G. *The Effect of Na-DMDT on Various Oxidative Processes in Intact Cells, in Comparison with the Effect on the Acetate Oxidation*

Following this comparatively detailed study of the effect of the dithiocarbamyl compounds on the oxidation of acetate, it was natural to compare the effect on different oxidative processes, as measured on intact cells.

As a standard procedure for these investigations, it was decided to use a yeast suspension with 1 gram yeast fresh weight per liter, 50 μ M zinc sulfate in the phosphate buffer of pH 6.3, and 100 μ M Na-DMDT, added from the side arm.

Glucose Oxidation. — First of all, the effect of Na-DMDT on the respiration of glucose was investigated anew. This had to be done, to see if the difference in sensitivity found between the oxidation of acetate and glucose merely could be due to heavy metal impurities in the former. However, it was invariably found that, even if also a considerable inhibition of the glucose

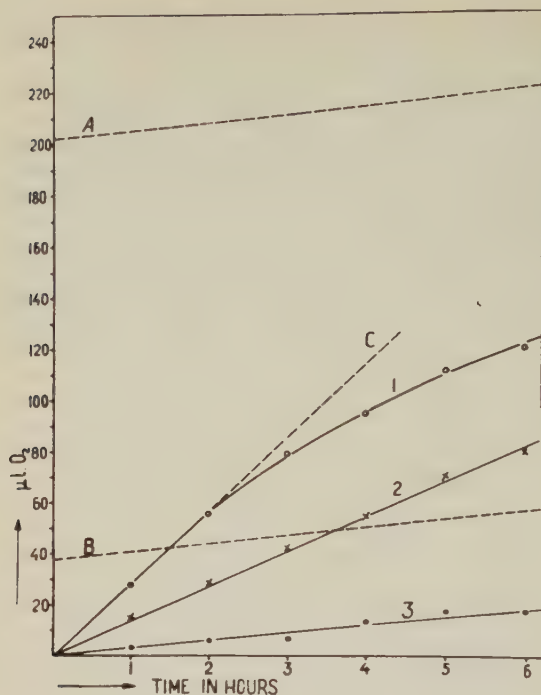


Figure 28. *The oxygen uptake of yeast suspensions with ethanol as the C-source. A total of 3 μ moles was added to each flask at zero time. Curve 1, without Na-DMDT. Curve 2, with 100 μ M Na-DMDT. 50 μ M zinc sulfate in the substrate. Line A denotes the theoretical oxygen uptake needed for complete oxidation of the added ethanol to carbon dioxide and water, while the line B denotes the amount needed for oxidation to acetaldehyde. Line C indicates the initial oxidation velocity of curve 1.*

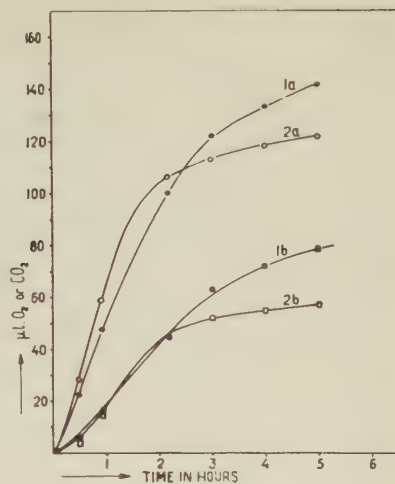
oxidation occurred under the conditions now used, the inhibition of the acetate oxidation was stronger. The results from these and the following experiments are compiled in table 7.

The Anaerobic Fermentation of Glucose. This was in the same medium found to be inhibited to the same, or a somewhat higher degree than the aerobic oxidation (table 7.).

Ethanol, Acetaldehyde Oxidation. An attempt was made to compare the inhibition of the oxidation of ethanol, acetaldehyde and acetate. It was found difficult to use acetaldehyde in the Warburg flasks, as it distilled into the alkali-cup, where it condensed to form a brownish product. This series therefore had to be omitted. But the oxidation of ethanol was found to be less inhibited than the oxidation of acetate. In this experiment, the substrate concentration was 0.1 *M*, and as the possibility existed that the ethanol was oxidized only to acetaldehyde, the experiment was repeated with a total of 3 μ moles ethanol in each flask, added from the side arm. Oxidation to acetaldehyde would then cause an oxygen uptake of 33.5 μ l, whereas the complete oxidation to carbon dioxide and water would need 202 μ l.

Figure 28 shows an oxygen uptake considerably in excess of the first value, indicating a complete oxidation of the ethanol. The inhibition in this

Figure 29. The oxygen uptake and carbon dioxide evolution of yeast suspensions to which have been added a total of 3 μ moles ethanol per flask. The yeast suspension in this particular experiment contained 2 grams per liter, 50 μ M zinc sulfate in the substrate. Curves 1 a and 2 a, oxygen uptake with and without 20 μ M Na-DMDT. Curves 1 b and 2 b, carbon dioxide evolution with and without 20 μ M Na-DMDT.



experiment, if maximal oxidation ratios are used for the calculation, is 59 per cent, agreeing with the values found with higher concentrations of ethanol (table 7).

As another method of following the steps in the ethanol oxidation from the level of acetic acid and onwards, the evolution of carbon dioxide was determined. In this experiment slightly different conditions were used, viz. 2 grams of yeast per liter, and 20 μ M Na-DMDT, 50 μ M zinc sulfate. The curves in figure 29 show a slight lag in the CO_2 evolution compared with the oxygen uptake, indicating the time needed for accumulation of a sufficient amount of acetic acid to start the further oxidation. It should be noted from this experiment that there is not a significantly greater inhibition of the carbon dioxide evolution than there is of the oxygen uptake, indicating that except for the first link in the oxidative sequence, little or no inhibition has occurred.

From the various findings reported in the literature regarding the mechanism of the ethanol oxidation, little doubt can exist about acetic acid or acetyl coenzyme A as an intermediate. The striking difference in sensitivity of the acetate oxidation to Zn-DMDT, depending on whether the acetate is supplied externally or formed internally, is very remarkable.

An interesting observation to be made from the curves in figure 29 is that the reaction inactivation which occurs normally under the conditions used here has been partially counteracted by Na-DMDT. The same observation was made with the oxidation of lactic acid (see later). TMTD did not affect the oxidation of ethanol more than Zn-DMDT.

Succinic Acid Oxidation. The oxidation of succinic acid was followed under the standard conditions. In spite of the high pH value, which is

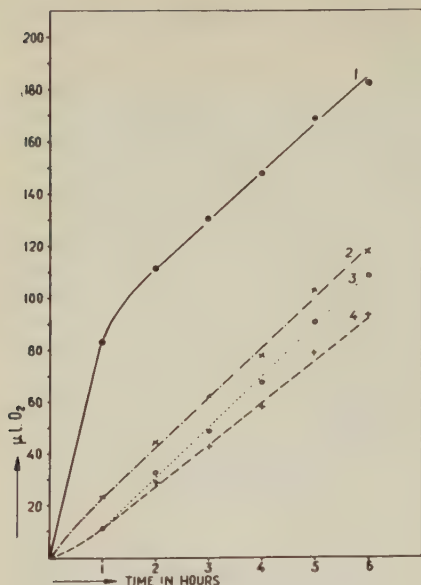


Figure 30. Oxidation of ascorbic acid by a yeast suspension in phosphate buffer of pH 6.2. Sodium ascorbate added from the side arm at zero time, to give a final concentration of 0.1 *M*. Curve 1, control curve with intact cells and without Na-DMDT. Curve 2, intact yeast cells, 100 μ M Na-DMDT added to the main compartment before the ascorbate. Curve 3, boiled yeast suspension. Curve 4, boiled yeast suspension to which has been added 100 μ M Na-DMDT.

unfavourable for the permeation of succinic acid, a slight but definite increase in respiration above the endogenous was observed. The differences were too small, however, to permit calculation of the degree of inhibition with certainty, but the results given in table 7 indicate that the inhibition is at least very slight.

Ascorbic Acid Oxidation. The enzyme ascorbic acid oxidase has been reported to be present in yeast by Tadokoro and Sakurai (Dawson and Tarpley, 1951). This is somewhat peculiar, since fungi have been reported to lack ascorbic acid.

In the present experiments, rapid oxygen uptake invariably followed the addition of sodium ascorbate to a yeast suspension of pH 6.2 in phosphate buffer. The oxygen uptake curves, of which curve No. 1 in figure 30 is representative, show the characteristics for the enzymic oxidation of ascorbic acid: A rapid initial reaction rate, soon followed by inactivation of the enzyme. After boiling, the initial rapid oxidation rate disappears (curve 3 in the same figure), and it would be natural to ascribe the linear rate obtained to non-enzymatic oxidation.

However, more detailed investigations have made the author very doubtful as to whether these curves actually demonstrate the presence of ascorbic acid oxidase in yeast. In these investigations, it was found that when to the phosphate buffer was added 10 μ M cupric sulfate, a curve similar to No. 1 in fig. 30 was obtained with intact yeast suspension. In boiled yeast suspen-

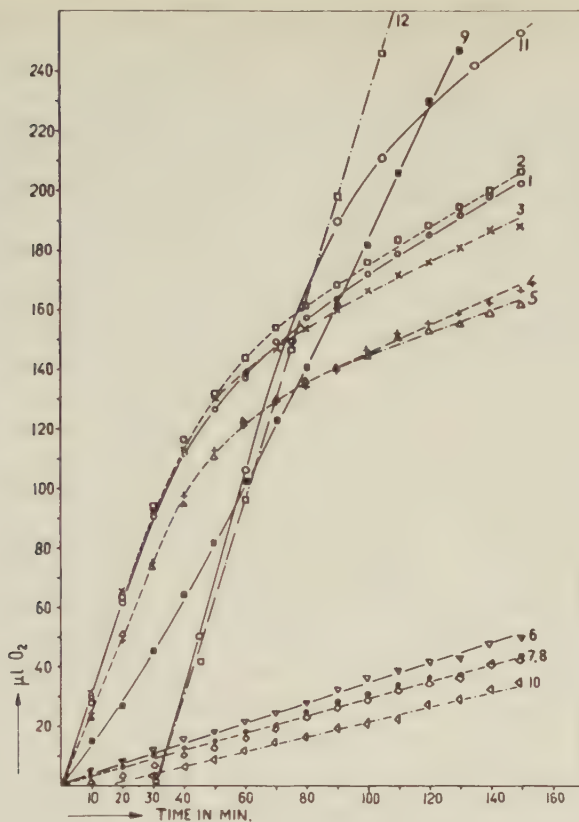


Figure 31. The oxidation of ascorbic acid by yeast suspensions in phosphate buffer of pH 6.2, to which has been added $10 \mu\text{M}$ cupric sulfate per liter, and $20 \mu\text{M}$ zinc sulfate. Sodium ascorbate added from the side arm at zero time, except for curves 11 and 12, where it was added after $\frac{1}{2}$ hour, to a final concentration of 0.1 M . Curve 1, control with intact yeast and no Na-DMDT. Curves 2, 3, 4, 5, 6, 7, 8, intact yeast with respectively 1, 2, 5, 10, 30, 50, and $100 \mu\text{M}$ Na-DMDT. Curve 9, boiled yeast suspension with no Na-DMDT. Curve 10, boiled yeast with $100 \mu\text{M}$ Na-DMDT. Curves 11 and 12 represent a second experiment with a new yeast suspension, but otherwise the same conditions. Curve 11, intact yeast suspension. Curve 12, the phosphate buffer with cupric sulfate and zinc sulfate only.

sion and in the phosphate buffer alone, oxygen uptake curves were then obtained, which at the start had a somewhat lower rate than in the presence of intact yeast. But as these were linear, or with a slightly increasing rate, the oxygen uptake velocity after about 1 hour and onwards was considerably higher than in the intact yeast suspensions (Figure 31).

The only possible explanation seems to be that in the intact yeast suspen-

sions, the copper compounds catalyzing the oxidation of ascorbic acid are after some period of time unavailable for this reaction. But this does not necessarily mean that these compounds are enzymes. It seems very difficult on the basis of the curves in figures 30 and 31 to conclude anything regarding the catalytic nature of the ascorbic acid oxidation, as it is difficult to predict in a cell suspension how much of the copper content under different conditions and after various treatments actually is available for the direct catalytic oxidation, and accordingly, how much of the oxidation rate must be ascribed to catalytic activities stronger than those of the simpler cupric complexes. The method of studying whether or not hydrogen peroxide is formed during the oxidation is not usable, owing to the catalase content of the yeast cells.

It should briefly be mentioned that Na-DMDT in low concentrations inhibited the oxidation of ascorbic acid completely, both the seemingly enzymatic oxidation illustrated in figure 30, and the obviously non-enzymatic one in figure 31.

Polyphenol Oxidation. Attempts were made to determine the oxidation of L-tyrosine and catechol by yeast suspensions. With catechol, a slight and steady, most probably non-enzymatic oxygen uptake was recorded. It was not affected by Na-DMDT in concentrations of 100 μM and below. With L-tyrosine, a slight oxygen uptake in excess of the endogenous respiration was also found, and again here it was unaffected by Na-DMDT. Results are seen in table 7. It should be recalled that James and Garton (1952) found 85 per cent inhibition of fungal polyphenolase by 200 μM Na-DEDT.

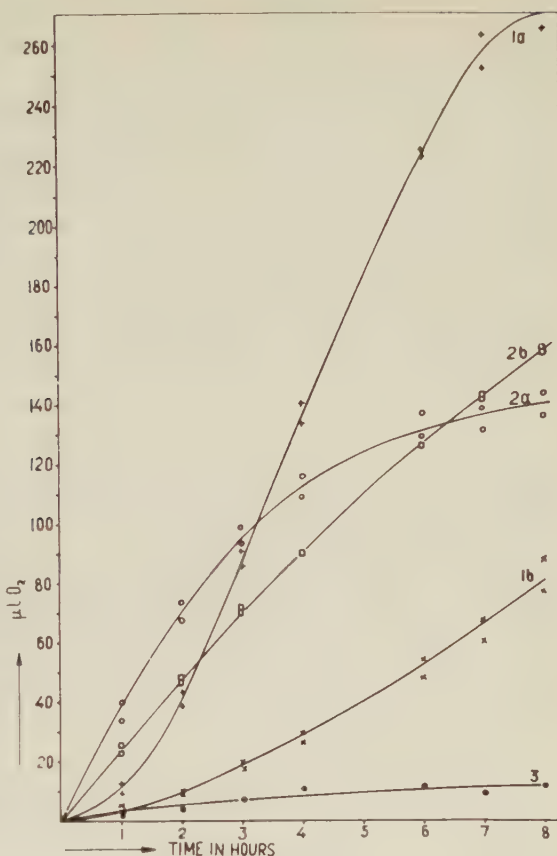
Lactic Acid Oxidation. When lactic acid is added to a yeast suspension, a fairly rapid oxygen uptake takes place. The first step in the oxidation leads to pyruvic acid, which further is oxidized to carbon dioxide and water by the normal respiratory route.

The lactic acid dehydrogenase belongs to the small and interesting group of dehydrogenases which are directly coupled with cytochrome c. By the early workers in this field (Dixon and Zerfas, 1939, Bach, Dixon and Keilin, 1942, Bach, Dixon and Zerfas, 1942), it was found that a haem-protein, by them called cytochrome b_2 , was an essential part of the enzyme system. Due to the instability of their preparations, however, they were not able to demonstrate conclusively whether the haem-protein also carried the dehydrogenase activity, or whether it was an essential intermediate carrier.

Recently, Appleby and Morton (1954) have prepared highly purified and crystalline cytochrome b_2 , and shown that the haem group (protohaem) is bound to the same protein molecule which has the lactic acid dehydrogenase activity. The prosthetic group for the latter is by them tentatively concluded to be a flavin mononucleotide.

The effect of Zn-DMDT on this rather unique enzyme was studied under

Figure 32. *The oxygen uptake of a yeast suspension with sodium lactate (50 μ moles per flask), or sodium acetate (10 μ moles per flask) as the C-source. 50 μ M zinc sulfate in the substrate. Curve 1 a, sodium acetate alone. Curve 1 b, sodium acetate with 100 μ M Na-DMDT. Curve 2 a, sodium lactate alone. Curve 2 b, sodium lactate with 100 μ M Na-DMDT. Curve 3, endogenous oxygen uptake.*



the normal conditions. Parallel experiments were done with acetate to compare the degree of inhibition. In some of the experiments only oxygen uptake was measured, and in others both oxygen uptake and carbon dioxide evolution (in order to follow the further oxidation of pyruvic acid). In calculation of the carbon dioxide evolution, no correction for the retention was made. The parallel experiments with acetate indicated, however, a retention of about 20 per cent (RQ calculated to 0.80—0.83 instead of the theoretical value 1.0).

In figure 32 the curves for the oxygen uptake following the addition of 50 μ moles sodium lactate per flask are compared with those for oxygen uptake following the addition of 10 μ moles sodium acetate, with and without Zn-DMDT. It is seen that Zn-DMDT inhibits the oxidation of lactate to a considerably smaller degree than it does the oxidation of acetate. As an interesting fact it is noted that the decline in activity in the control flasks

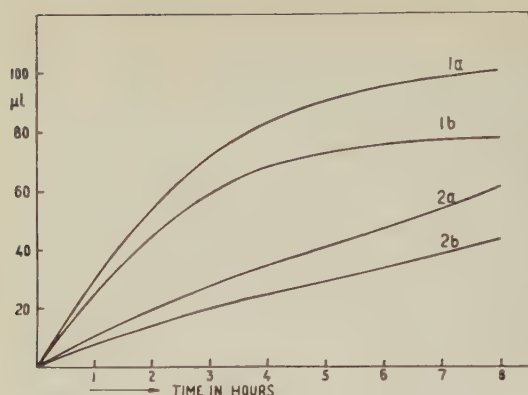


Figure 33. The oxygen uptake and carbon dioxide evolution of a yeast suspension with sodium lactate (50 μ moles per flask) as the C-source. 50 μ M zinc sulfate in the substrate. Curves 1 a and 2 a, oxygen uptake without and with 100 μ M Na-DMDT. Curves 1 b and 2 b, carbon dioxide evolution without and with 100 μ M Na-DMDT. All curves corrected for endogenous respiration.

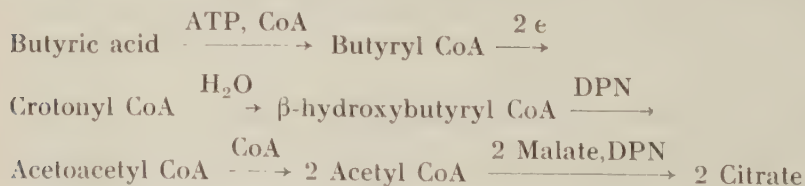
(probably due to a reaction inactivation) has disappeared after the addition of the dithiocarbamate, so that after 4 hours, the rate in the controls is lower than when Zn-DMDT is present.

Figure 33 shows that the carbon dioxide evolution would correspond to a slightly slower oxidation of pyruvic acid than the velocity at which it is formed from lactic acid. But this difference may also be due to retention. In any case, the carbon dioxide evolution has not been inhibited further than the oxygen uptake, showing that no significant inhibition of the oxidation of pyruvic acid has occurred.

D-amino Acid Oxidation. Attempts to add D-alanine to intact yeast suspensions did not result in increased oxygen uptake above the endogenous respiration.

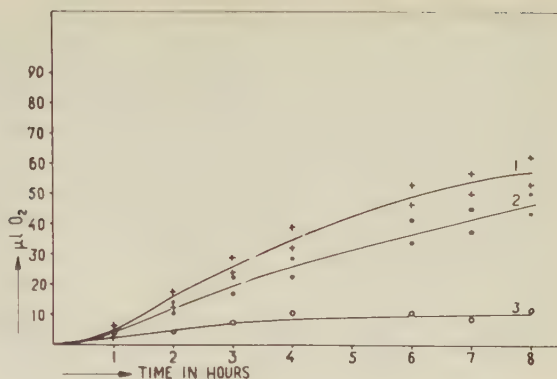
Glutamic Acid Oxidation. Likewise, glutamate addition gave so small an increase in the oxygen uptake above the endogenous, that no safe conclusions regarding the effect of Zn-DMDT could be drawn.

Butyric Acid Oxidation. The oxidation of fatty acids in animal liver and in certain bacteria has been clarified in the last couple of years. The sequence of breakdown of butyric acid has been shown to be as follows in animal tissues (Beinert et al, 1953):



Of the various enzymes catalyzing this sequence, the butyryl CoA dehydrogenase would be of particular interest in the present work. As it has been shown to be a cupro-flavoprotein (Mahler, 1953, Mahler, 1954), and since it

Figure 34. The oxidation of butyric acid by a yeast suspension. Curve 1, control, without Na-DMDT. Curve 2, with 100 μ M Na-DMDT. Curve 3, endogenous oxygen uptake. 50 μ M zinc sulfate in the substrate.



plays a very definite and central role in the metabolism, it would be interesting to see if Na-DMDT were able to inhibit it.

It was found that when Na-butyrate was added to a yeast suspension, a significant increase in the oxygen consumption above the endogenous occurred. It was further repeatedly found that Na-DMDT in concentrations of 100 μ M and below did not to any appreciable degree inhibit the oxidation. Curves from one experiment with a relatively high degree of inhibition are seen in figure 34. Further data are found in table 7.

VII. The Effect of the Dithiocarbamyl Compounds on the Pasteur Mechanism in Intact Cells

The energy liberated by the various oxidative steps in the carbohydrate breakdown is transferred to phosphate bonds by means of a reaction scheme which just recently has been partially evaluated (Boyer, Falcone, and Harrison, 1954).

There is much biochemical evidence supporting the assumption that the levels of inorganic and energy-rich phosphate act as regulators of the reaction rate of energy-generating processes in the cell. With liver mitochondria it has been definitely shown that the respiratory rate decreased when the inorganic phosphate was transferred to, and allowed to accumulate as energy-rich phosphate, whereas the respiratory rate increased when to the system were added enzymes which consumed the energy-rich phosphate. (Lardy and Wellman, 1952.) This would lead to a very straightforward connection between oxidative phosphorylation and the *Pasteur effect*. Such a direct connection does not seem to be acceptable to several authors (see Dickens, 1951 and Fruton and Simmonds, 1953, pp. 483—486). However,

Table 8. *The effect of dithiocarbamyl compounds on the Meyerhof coefficient (MQ). Experiments made in pure nitrogen and air, with yeast suspensions in 0.02 M phosphate buffer of pH 6.0 containing 2.5 % glucose. The gas exchanges given in μ liters as the amounts actually determined during the experiments.*

Compound added	Heavy metal addition	N ₂ q _{CO₂}	Air q _{O₂}	Air q _{CO₂}	MQ
None	None	102.0	48.0	76.0	1.54
Na-DMDT, 10 μ M ...	None	88.0	46.5	50.5	1.81
Na-DMDT, 100 μ M ...	None	92.5	47.0	69.0	1.50
PNP, 10 ⁻³ M	None	104.5	51.0	138.0	0.34
None	20 % salt solution	234.0	124.0	143.5	1.73
Na-DMDT, 100 μ M ...	do.	123.5	78.0	81.0	1.55
TMTD 100 μ M ...	do.	165.0	79.0	124.0	1.52

whether the connection is simple or more complicated, as for instance via a resynthesis of carbohydrate, the fact that the same type of inhibitors affect oxidative phosphorylation and the Pasteur effect (Meyerhof and Fiala, 1950) would mean that when the link between oxidation and phosphorylation is broken, the Pasteur mechanism is also prevented from operating.

A study of the effect of dithiocarbamyl compounds on the Pasteur effect would therefore be expected to furnish information as to whether these compounds interfere with the oxidative phosphorylation or not.

A determination of the Pasteur coefficient, or its equivalent, the Meyerhof coefficient (MQ) can easily be made on intact cells, whereas estimation of phosphorylative P/O ratio is a procedure needing isolation of particular cellular systems. Only determinations of MQ have, therefore, been done in this work.

Fresh yeast, treated as described in the preceding chapter, was used in these experiments.

The anaerobic Warburg flasks were flushed with nitrogen from a steel cylinder. Traces of oxygen were removed by passing the nitrogen through a copper oven previously reduced with gaseous hydrogen. To check the anaerobic conditions, alkali was placed in the center cup of one of the flasks containing nitrogen, so that a possibly occurring oxygen uptake might be detected. Only a negligible oxygen uptake was found if the nitrogen was passed through the copper oven. If this process was omitted, a considerable respiration occurred, rendering the experiment valueless.

The aerobic flasks contained air. The concentration of glucose was 2.5 per cent. The dithiocarbamyl compounds were tipped in from the side arm at zero time, except for TMTD, which was added by the method described on p. 772 was used.

The results from a number of experiments are summarized in table 8. The data are given in the form of Meyerhof coefficients:

$$MQ = \frac{\text{Mol CO}_2 \text{ fermentation suppressed by oxidation}}{\text{Mol O}_2 \text{ consumed}}$$

MQ is one third of the Pasteur coefficient, and the maximum experimental value generally obtained is around 2.0.

Control experiments were made with *p*-nitrophenol (PNP), which is known to be a potent inhibitor of the Pasteur effect (Meyerhof and Fiala, 1950).

The results show conclusively that the dithiocarbamyl compounds have no effect at all on the phosphorylative couplings and the energy transfer in intact cells at concentrations which cause growth inhibition.

VIII. The Effect of the Dithiocarbamyl Compounds on the Anabolic Processes

A. Methods

For growth, the nutrient solution earlier used as a »model» solution (see p. 738) had to be modified. First of all a vitamin mixture containing the B vitamins which according to Robinson (1951) are essential for growth of *S. cerevisiae* was added in order to obtain growth at all. This vitamin mixture consisted of:

Aneurin HCl	50 mg
Ca pantothenate	100 mg
Pyridoxin	100 mg
Inositol	100 mg
Biotin	10 mg
Distilled water to 1000 ml	

With 20 ml of this solution added per liter of nutrient solution, a satisfactory growth was obtained. No study was made of whether the mixture was optimal as regards the various growth factors.

The nutrient solution was also diluted and prepared in the following manner: 200 ml of 1, 100 ml each of 2 and 3 (pH 6.3), 20 ml of the vitamin mixture, 10 grams of glucose, and distilled water to 1000 ml.

When this mixture was used, no precipitation occurred, so that boiling and filtering were not necessary. But it was found that the solution contained too much copper for obtaining an optimal growth. Therefore, solution 1 had also to be prepared with distilled water instead of tap water. (The effect of supra-optimal concentrations of copper is very easily discovered in the experiments with Na-DMDT, as it causes a distinct growth stimulation in the inversion area.)

The growth experiments were carried out in the constant temperature bath previously described, at a temperature of 30° C., and with slow aeration all the time. Each flask contained 200 ml of the nutrient medium. Samples of 10 ml were taken out at suitable time intervals for analysis. In a few cases, growth experiments were also carried out in the Warburg apparatus, for comparison of growth and respiration.

Because of the heavy inoculum used in these experiments, and the short duration (5—8 hours), no precautions against infections were taken. In microscopic examinations, no signs of significant bacterial contamination were ever found.

The growth was normally followed photometrically, with a Lumetron photometer, using filter M 620 and measuring the transmitted light. An extinction curve

was prepared by suspending a weighed amount of yeast in distilled water and diluting it to suitable concentrations. The extinction curve was practically linear below values of 0.8 (corresponding to 0.75 g yeast per liter). No change in extinction values could be observed if the suspensions were prepared with a 2.5 per cent glucose solution instead of water.

In experiments starting with a concentration of yeast as high as 0.5 grams per liter fresh weight, all samples were diluted with an equal volume of distilled water before the turbidity was measured. According to Steen (1954) the turbidity of bacteria is most closely correlated with the dry weight of the bacterial suspension, the correlation with nitrogen content or bacterial count being considerably less.

In some experiments the growth was also followed by counting. The samples used for this were the same that were used for turbidity measurements. 2 ml formalin was added to the 10 ml samples, and they were kept in a refrigerator until counted, at most until the next day. A Bürker counting chamber was used, and the cells in 10 or 20 of the $\frac{1}{25}$ mm² squares counted, according to the number in each square. The total count in each sample varied between 200 and 500 cells. The data obtained were used for drawing growth curves, and the interrelationship between the figures therefore reduces the probable error in each counting. Since the results obtained were quite clear-cut, and only a qualitative discussion necessary, the data have not been subjected to statistical treatment.

pH was determined during the growth on the same samples taken out for the turbidity measurements, by means of a Metrohm model E 187 potentiometer with a combined electrode. The main purpose of the pH determinations was to insure that the pH did not fall below »permissible» values during growth. It was found that under the conditions used for the growth experiments reported here, pH never fell below 5.8 (from a starting value of 6.3). Since no observations of physiological interest have been derived from the pH values, they are omitted from this report.

In the cases where the dry weight of the yeast was determined at the end of the experiments, it was done on an aliquot portion of the suspension which was centrifuged, the supernatant sucked carefully off, the yeast cells resuspended in about 50 ml distilled water, centrifuged anew and finally transferred with a few ml of distilled water to weighed beakers, which were evaporated to dryness at 100° C. It can be calculated that one washing with 50 ml of distilled water is sufficient to reduce the dry-matter content in the liquid following the cells to less than 0.2 mg per ml.

Nitrogen was determined according to Kjeldahl, with selenium as catalyst during the digestion. The digestion was started in the beakers used for weighing, by adding 5 ml of a solution consisting of 50 per cent sulfuric acid with 0.1 per cent cupric sulfate and a trace of selenium dioxide, and heating them carefully. The contents were transferred, by as little distilled water as possible, to Kjeldahl flasks when dissolved, but before carbonization had started.

Glucose was determined by the iodometric method of Auerbach and Bodenländer (1923).

Acetate uptake was determined by using sodium acetate-1-C¹⁴. A sample containing 1 mC per mmole was diluted with inactive acetate to obtain radioactivities lying in the order of magnitude of 1 μ C in each experiment. In all acetate fixation experiments, the acetate concentration was 2 mM, and the glucose concentration 1 per cent. The radioactivity was determined in the various samples by pipetting out portions containing not more than 5 mg dry matter onto copper or glass discs, and

evaporating them to dryness. When total radioactivity including that in the acetate was to be determined, sodium hydroxide was added to the disc before the evaporation. When radioactivity in other compounds than acetate was to be determined, hydrochloric acid was added before evaporation. It was found impossible to evaporate trichloroacetic acid (TCA) extracts in copper discs, owing to the strong attack by TCA on copper, which resulted in a severe loss of counts owing to self-absorption in the mixture of the sample and copper salts in the disc. In this case, glass discs prepared from thin-walled glass tubes were found to be very convenient. They can be made by a glass-blower by means of a cutting machine for the same price as copper discs, and are probably preferable in a number of cases.

The radioactive yeast was fractionated according to a slight modification of a procedure by McQuillen and Roberts (1954). After the end of growth, the yeast was centrifuged, washed once with distilled water and resuspended in 5 ml distilled water. 1 ml was transferred to copper discs and evaporated with 0.5 ml 0.2 *M* hydrochloric acid, for determination of the total nonvolatile radioactivity. To the rest of the suspension was added 4 ml cold 10 per cent TCA. After half an hour in the refrigerator, centrifugation and removal of the solution samples of 1–2 ml of the TCA extracts were transferred to glass discs and evaporated. This fraction (fraction No. 1) contains amino acids and a number of other lowmolecular weight compounds. The residue was first treated with 75 per cent ethanol, centrifuged, and then treated with a mixture of equal volumes of 75 per cent ethanol and ether. These extracts were made up to a total volume of 25 ml, and 2 ml were transferred to copper discs. The combined alcohol-ether fraction (fraction No. 2) contains mainly lipides, but probably also some proteins which are soluble in 75 per cent ethanol.

The residue was boiled with 5 ml 0.1 *M* sodium hydroxide for about 10 minutes. The proteins and nucleic acids now go into solution, and are obtained in fraction No. 3. Of this, 1 ml samples were transferred to copper discs for evaporation. The residue was washed with a little distilled water (discarded), and transferred quantitatively to copper discs. This residue (fraction No. 4) probably for the major part contains cell-wall material such as polysaccharides and chitin.

The radioactivity in the various samples thus obtained, was determined by counting with a thin-walled Geiger-Müller tube, connected with either a Tracerlab Superscaler or Autoscaler.

B. The Effect of Na-DMDT on Growth, Respiration of Growing Cells, Glucose Consumption and Acetate Fixation

Since an actual demonstration of the growth-inhibiting effect of the dithiocarbamyl compounds on yeast has been omitted from the introductory parts of this work, where such a demonstration perhaps belonged, some growth experiments will be discussed a little in detail first. These experiments were carried out to study the effect of the concentration of the initial inoculum of yeast on the growth rate and the action curve for Na-DMDT. Figure 35 shows the growth as a function of time. It is seen that increase in turbidity only starts after a lag period of about 2 hours. This lag period was observed in all experiments. If, however, attention is turned towards figure 37, it can

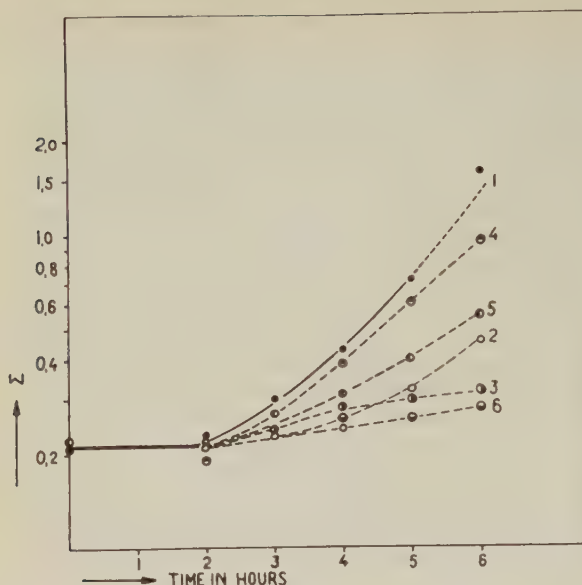


Figure 35. The growth of yeast in a nutrient solution to which varying concentrations of Na-DMDT have been added, measured turbidimetrically. Start inoculum, 0.25 grams per liter. Curve 1, no Na-DMDT. Curve 2, 1 μ M. Curve 3, 2.5 μ M. Curve 4, 6.25 μ M. Curve 5, 15 μ M. Curve 6, 50 μ M.

be seen that when cell counts are taken as a measure of the growth, the lag period is only about 1 hour. Probably the cells first start to divide with no increase in total volume. After a while, the volume of the individual cells perhaps also starts to increase. The opposite sequence was observed by Steen (1954) on *Meningococcus*, and explained in an analogous manner by assuming that the bacteria first started to increase in size, and then to divide.

Returning to figure 35, it is seen that in the presence of Na-DMDT, the growth inhibition may vary considerably with time, as some of the curves show a decrease of inhibition, and others an increase.

In figure 36 are seen the action curves for Na-DMDT at varying start concentrations of yeast. In all curves, the inversion phenomenon is clearly shown. The first inhibition maximum is seemingly dependent on the yeast concentration, while no connection can be found for the second zone of inhibition. The explanation may also here, as in the case of acetate oxidation, be that the yeast carries traces of heavy metals on the cellular surfaces.

In one experiment the effect of adding Na-DMDT to cells while actively growing was studied. Because of the lag period, no information regarding the rapidity of the action of Na-DMDT could be obtained when it was added at the start of the experiment. In this case, cellular counts were also taken, in order to compare the two methods of determining growth. The results, shown in figure 37, are very interesting. It is seen that in the flasks where

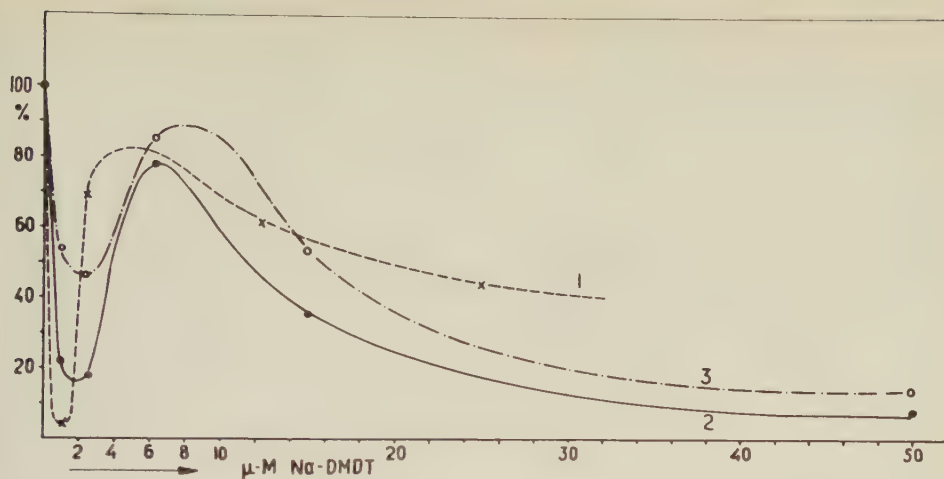


Figure 36. Growth of yeast after five hours, at varying concentrations of Na-DMDT, in per cent of the growth without Na-DMDT. Curve 1, start concentration of yeast 0.125 grams per liter. Curve 2, 0.25 grams. Curve 3, 0.50 grams per liter. Ordinate: extinction increase in per cent of that without Na-DMDT.

Na-DMDT has been added, cell division has stopped immediately, while the increase in turbidity has continued at a slowly declining rate. This would indicate an increase in the size of individual cells following addition of Na-DMDT. No volume determinations have been made in this work. Giant cells have never been observed as being caused by dithiocarbamyl compounds, but Loveless, Spoerl and Weisman (1954) state that cells of *S. cerevisiae* whose growth was inhibited by Na-DEDT have a volume 160 per cent of that of the controls. This substantiates the present findings.

The ability to assimilate acetate was also compared with growth, glucose consumption and respiration. In the first of the experiments to be reported here, the acetate fixation was compared with the growth and the glucose consumption in normal and growth-inhibited cells. The curves are seen in figure 38. It can be seen that the glucose consumption and acetate fixation follow the growth very closely, the latter being somewhat more inhibited than the former. In the next experiment, which was made in the Warburg apparatus, growth and acetate fixation were compared with the respiration. The results are summarized in table 9. It is seen that even in cultures where the growth has been completely arrested the relative oxygen uptake (measured as the quotient of oxygen uptake during a period of actual growth, and the turbidity at the end of this period) is remarkably constant. Further, it is noteworthy that the amount of tracer carbon derived from acetate is closely

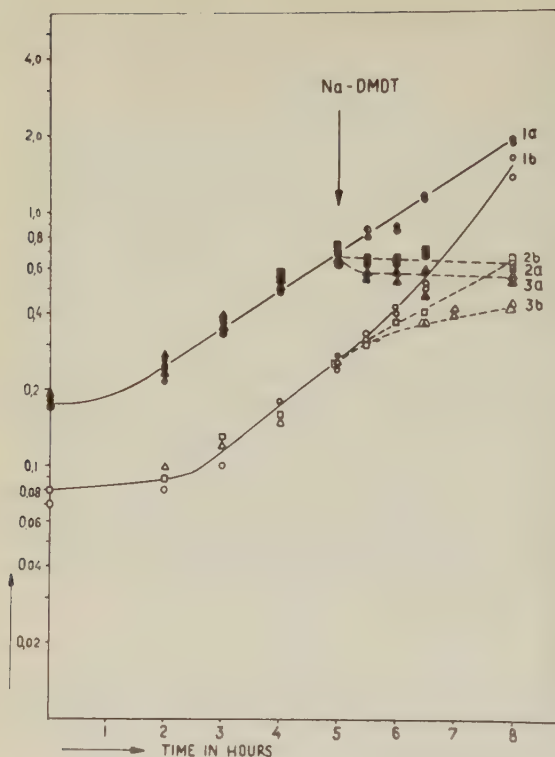


Figure 37. Growth of yeast in a nutrient solution with varying concentrations of Na-DMDT, added after 5 hours (indicated by arrow). The growth was followed by counting (upper curves, filled symbols), and by turbidity measurements (lower curves, open symbols). Curves 1, without Na-DMDT. Curves 2, with 1 μ M Na-DMDT. Curves 3, with 50 μ M Na-DMDT. Ordinate: extinction values read directly on the scale; number of cells per ml found by multiplying ordinate values by 10^7 .

correlated with the degree of growth, and not with the total oxygen consumption.

In connection with these growth experiments, a comparison was made between the action curve for Na-DMDT on growth and on acetate oxidation, since the substrate had been considerably modified since the earlier acetate oxidation experiments. For the sake of simplicity, the comparison was carried out in a substrate where ammonium chloride and the vitamin solution were omitted, as was, of course, glucose. The curve obtained (figure 39) shows a striking similarity to the growth inhibition curves.

A number of investigators have studied the distribution of C^{14} from labeled acetate in yeast and other microorganisms (e.g. Gilvarg and Bloch, 1951, Wang, Labbe, Christensen and Cheldelin, 1952, Labbe, Thomas, Cheldelin, Christensen and Wang, 1952, Wang, Thomas, Cheldelin and Christensen, 1952, McQuillen and Roberts, 1954, Nossal, 1954c). Without going into details, it might be stated that acetate enters the Krebs cycle as acetyl coenzyme A and labeled carbon appears first in the Krebs cycle intermediates and in the amino acids derived directly from them. At later stages, acetate

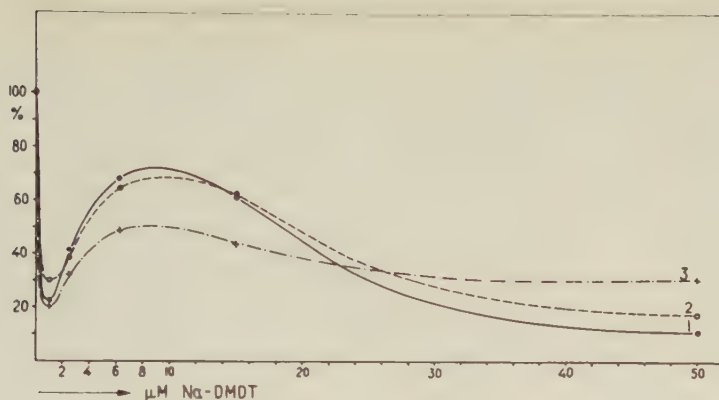


Figure 38. *Growth (measured turbidimetrically), glucose consumption and acetate fixation of yeast after five hours at varying concentrations of Na-DMDT. Curve 1, growth. Curve 2, glucose consumption. Curve 3, acetate fixation. All values given as percentages of those without Na-DMDT.*

carbon appears in nearly all compounds in the cell, such as proteins, peptides, lipides, nucleic acids and a variety of low-molecular weight compounds. Relatively small amounts of acetate carbon are lost as carbon dioxide.

An attempt was made in a series of experiments to fractionate further the radioactive compounds formed from acetate- C^{14} . Owing to the low activity obtained in growth-inhibited cultures, the results are rather uncertain. As mentioned, the method of McQuillen and Roberts (1954) was followed, with the exception that the fractions containing only very small amounts of activity (proteins soluble in 75 per cent ethanol and nucleic acids) were omitted, as the error in determination would in any case make it impossible to draw any conclusions. Table 10 gives the results from one of these experiments.

There is satisfactory agreement between the sum of the various activities and the amount found by direct measurement of a sample of the whole yeast, with the exception of culture No. 4. The degree of self-absorption has probably been slightly higher in the material from cultures 1—3 than in that from cultures 5 and 6.

In the first fraction, the cold TCA fraction containing low molecular weight compounds such as amino acids, peptides, prosthetic groups, etc., there is no difference in relative activity. In the next fraction, the alcohol-ether fraction containing lipides, the relative activity in the growth-inhibited cells is significantly higher than in the normal ones. This would indicate that a higher rate of lipid synthesis in relation to synthesis of other cellular material is found in growth-inhibited cells than in normal cells. In proteins,

Table 9. *The connection between the growth, measured turbidimetrically, and the oxygen consumption during the second hour of growth (end of the lag period), and the fifth hour of growth. Various concentrations of Na-DMDT. The experiment was carried out in the Warburg apparatus. Start concentration of yeast was 0.5 grams per liter. For measurements of turbidity, 2 ml of the suspension in each flask was diluted with 8 ml distilled water. The values read in the photometer are given in the table. The experiment lasted 5 hours.*

Warburg flask No.	1	2	3	4	5	6	7	8	9	10	11	12
Na-DMDT conc.:	0	0	0	0	0.2	0.4	1	2	4	8	12	20
Start extinct.	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07
End extinct.	0.32	0.32	0.31	0.34	0.34	0.24	0.09	0.07	0.06	0.09	0.13	0.18
Increase %	100				100	67	8	0	0	8	24	44
Respiration, μ l O ₂ up- take the second hour	32.5	36.0	34.0	34.5	29.5	28.0	27.0	27.0	26.5	24.5	29.0	26.5
% of control	100				87	82	80	80	78	72	85	78
Respiration, μ l O ₂ up- take 4—5 hours.....	74.0	78.0	74.0	75.0	69.0	64.5	36.5	28.0	24.0	30.0	46.0	42.5
Relative respiration in- tensity, μ l O ₂ /extinct.												
the second hour.....	46	51	49	50	42	40	39	39	38	35	41	38
the fifth hour	23	24	24	22	20	27	25	25	25	30	35	24
Total oxygen uptake, μ l												
% of 1—4	100				100	86.0	60.5	54.5	50	53	71	66
Acetate fixated. c.p.m.												
% of 1—4	100				111	57	4	0	0	10	27	58

which were extracted with warm 0.1-N NaOH, there is no difference in relative activity. The residue shows a distinctly lower relative activity in growth-inhibited than in normal cells. This residue is of a somewhat undefined constitution, but it must be believed to consist for the greater part of cell wall material, since it is insoluble in alkali (Houwink and Kreger, 1953). On residue samples 1—8, nitrogen determinations gave the values 4.6, 4.8 and 5.4 per cent N respectively. The residue must then also contain nitrogenous material — probably protein. If it is assumed that the residue is composed only of proteins and cell wall material, the protein content is 25–30 per cent of the total. The dry weight of the residue was approximately 10 per cent of the original dry weight of the yeast. Even though no nitrogen determinations could be made on samples 4—6, owing to the small amount of material, it seems permissible to ascribe the difference in activity of the residues from growth-inhibited and normal cultures to the part of the residue that is of a non-protein origin, probably cell wall material. The metabolic processes leading to cell wall synthesis should have been much more severely inhibited than the other anabolic processes, according to the results from

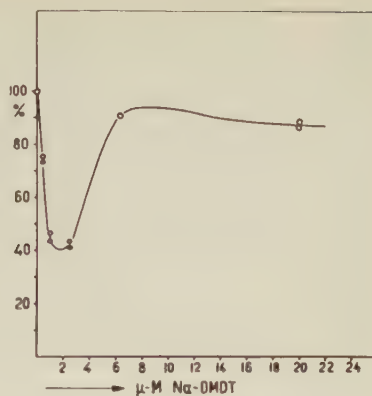


Figure 39. Oxidation of acetate by a yeast suspension in the nutrient medium used for the growth experiments, at varying concentrations of Na-DMDT. Ordinate: oxygen uptake in μ liters during five hours in per cent of that without Na-DMDT.

Table 10. Fractionation of radioactive material in yeast grown in the presence of acetate — $1\text{ }C^{14}$. Numbers 1—3 refer to control cultures, and the numbers 4—6 to cultures to which have been added $1\text{ }\mu\text{M}$ Na-DMDT. The ordinary substrate, with the addition of 2 mM Na acetate was used. Start pH was 6.3. Start concentration of the yeast was $0.125\text{ grams (fresh weight) per liter}$. Cultures 1—3 were taken after 7 hours, and cultures 4—6 after 10 hours of incubation at 30° C . The growth was followed turbidimetrically. After growth ended, the culture suspension was centrifuged, washed once with distilled water, resuspended and samples taken out for activity measurements. These samples were evaporated with HCl for removal of acetic acid. On the rest of the suspensions, a fractionation procedure was carried out, which is referred to in the text. Radioactivity measurements were made with a Tracerlab Autoscaler. 4096 counts were registered. The background was 35 counts/min.

Culture No.	1	2	3	4	5	6
Conc. Na-DMDT	—	—	—	$1\text{ }\mu\text{M}$	$1\text{ }\mu\text{M}$	$1\text{ }\mu\text{M}$
Start turbidity	0.07	0.08	0.08	0.08	0.07	0.07
After 7 hours, turb.	0.64	0.75	0.77	0.12	0.11	0.11
After 10 hours, turb.				0.14	0.13	0.12
Growth (increase in turb.)	0.57	0.67	0.69	0.06	0.07	0.05
Total activity directly measured, counts/min.	6,680 6,330	6,300 6,350	6,380 6,850	640 635	595 570	585 580
Cold TCA, c/min.	950	825	925	90	75	100
Alc.-ether, »	1,665	1,685	1,880	345	330	220
NaOH, »	1,775	1,710	1,760	222	117	163
Residue, »	1,425	1,600	1,750	95	28	55
Sum »	5,805	5,820	6,305	750	550	538
Activities in per cent of sum:						
Cold TCA	16.5 ± 1.0	14.0 ± 1.0	14.5 ± 1.0	12.0 ± 1.2	13.5 ± 1.4	18.5 ± 1.9
Alc.-ether	28.5 ± 0.3	29.0 ± 0.3	30.0 ± 0.3	46.0 ± 1.0	60.0 ± 1.5	41.0 ± 1.0
NaOH	30.5 ± 0.3	29.5 ± 0.3	28.0 ± 0.3	29.5 ± 0.3	21.0 ± 1.5	30.0 ± 1.5
Residue	24.5 ± 0.5	27.5 ± 0.5	28.0 ± 0.5	12.0 ± 0.5	5.0 ± 0.2	10.0 ± 0.4

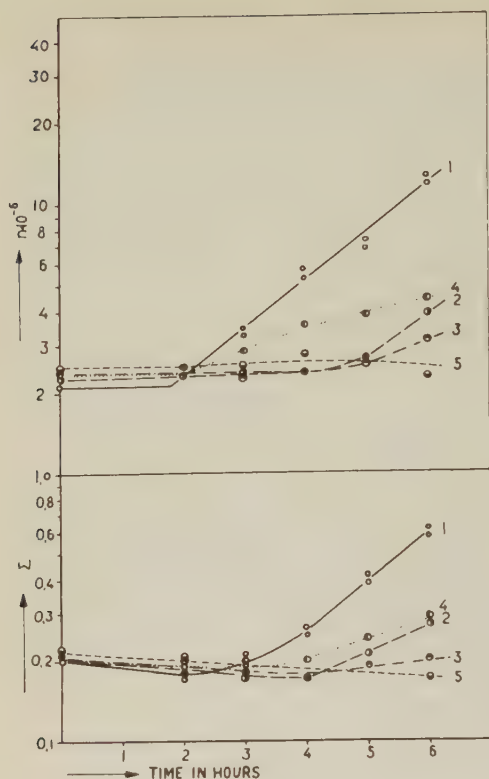


Figure 40. Growth curves from experiment No. 3 in table 8. Upper curves: cell counts (ordinate, cell number per ml, multiplied by 10^{-6}). Lower curves: turbidity measurements (ordinate: extinction value). Curves No. 1, no Na-DMDT. Curves No. 2, $0.5 \mu M$. Curves No. 3, $1 \mu M$. Curves No. 4, $5 \mu M$. Curves No. 5, $10 \mu M$. Curves No. 6, $50 \mu M$ Na-DMDT.

this experiment, and if these considerations are correct. The weakness of the experiment should, however, be stressed. It is only by analogy that the fractionation of the growth-inhibited yeast is assumed to give the same division of the cell-material as the fractionation of normal cultures. Such a conclusion is probably permitted in the case of the two first fractions, but the amount dissolved in NaOH might be dependent on a number of factors correlated more or less directly with the growth inhibition. A very careful examination would be needed before experiments of this type may yield conclusive results.

C. Nitrogen Assimilation

From the tracer experiments reported in the preceeding section, the conclusion might be drawn that both the lipide and the protein metabolism proceed quite normally in growth-inhibited cells.

Additional evidence in favour of the protein metabolism being unaffected by the dithiocarbamyl compounds in growth-inhibited cells could be obtained by preparing yeast low in nitrogen, and studying its behavior when trans-

Table 11. *Dry weight* (total in mg. per culture flask) *and nitrogen content in per cent of the dry weight, of yeast pretreated to lower the nitrogen content by being shaken overnight in a nutrient medium without N, and then grown in an ordinary medium with varying concentrations of Na-DMDT* (in the table given as μ moles per liter), *for six hours.*

Exp. no.	Flask no.	1	2	3	4	5	6
1	Na-DMDT conc.	0	0	1	1	50	50
	Start weight ...	27.9	27.9	27.9	27.9	27.9	27.9
	End weight	60.2	86.3	33.0	32.7	31.7	26.3
	% N at start ...	3.43	3.43	3.43	3.43	3.43	3.43
	% N at end	9.20	9.25	8.40	7.35	6.25	5.85
2	Na-DMDT conc.	0	0	1	1	50	50
	Start weight ...	27.3	27.3	27.3	27.3	27.3	27.3
	End weight	104.5	107.5	26.0	26.0	38.8	42.3
	% N at start ...	3.53	3.53	3.53	3.53	3.53	3.53
	% N at end	9.60	10.70	5.90	5.50	11.80	11.80
3	Na-DMDT conc.	0	0	0.5	1	10	50
	Start weight ...	26.0	26.0	26.0	26.0	26.0	26.0
	End weight	101.5	105.0	48.0	28.8	51.4	21.9
	% N at start ...	3.84	3.84	3.84	3.84	3.84	3.84
	% N at end	10.20	9.80	9.60	8.40	9.00	8.60

ferred to a normal substrate with dithiocarbamate present. Under such conditions, it would be expected that nitrogen assimilation processes could operate independent of the process of cellular growth and division.

The physiology of microorganisms with artificially lowered N-content has been studied by Virtanen and collaborators in a number of papers. (Virtanen and De Ley, 1948, Virtanen, 1948, Virtanen and Winkler, 1949, De Ley, 1949, Virtanen, 1949). Generally, it may be stated that in such microorganisms the adaptive enzyme systems have disappeared, while the constitutive remain.

The yeast used for these experiments was starved on nitrogen by being shaken overnight in a complete nutrient medium without N. The nitrogen content, which on dry weight basis in the purchased yeast was around 6.5—7 per cent, decreased by this treatment to around 3.5 per cent. The cells were then transferred to a normal nutrient substrate with ammonium chloride to which varying concentrations of Na-DMDT were added. The growth was followed turbidimetrically; in one experiment it was followed by counting. Growth curves from this experiment are seen in figure 40. It is seen that the lag period is longer than normal; otherwise the growth is similar to that in ordinary yeast.

At the end of the experiment aliquot portions of the suspensions were centrifugated, the yeast washed and the dry weight determined. The same was done with an aliquot of the inoculation suspension. The nitrogen content was subsequently determined in the inoculum and in the yeast taken at the end.

The results from three successive experiments are summarized in table 11. They show that even in yeast which has been completely inhibited in growth, the accumulation of nitrogenous substances has proceeded so that the N-level has become fully normal.

It should thus be reasonable to conclude that growth inhibition has nothing to do with nitrogen assimilation as a whole.

IX. Some Observations Regarding the Effect of Dithiocarbamyl Compounds on Enzyme Reactions

A. Introduction

Studies of the effect of biocidic substances on enzyme reactions might have two purposes. One would be to investigate, using highly purified enzymes, the mode of action of the compound when inhibiting a particular enzymic reaction, and to explain this in physico-chemical terms. The other purpose would be to avoid the difficulties encountered by the cellular membrane, and study the action of the compound, at a deliberately chosen concentration, on an enzyme system or on an enzyme sequence selected, but still operating in the cytoplasmic soup obtained by homogenization. Studies in these directions can give no evidence as to the mechanism of action of the compound when acting on living isolated organisms, but they may furnish decisive support to studies with intact organisms, and strengthen the evidence obtained in such work.

In the present work, only a few experiments have been done with cell-free homogenates, and none with purified enzyme systems. The reason for this was (as shall presently be reported) that from the experiments with succinic oxidase the conclusion may be drawn that dithiocarbamyl compounds do not enter the interior of the cells. It would of course still be of great theoretical interest to collect information about the action of these compounds on various enzyme systems, but such studies would have little value if not carried out with purified systems, and the subject is far too extensive for inclusion in the present work.

B. Succinic Oxidase

For homogenization, about 5 grams of fresh baker's yeast was suspended in a little distilled water and spread on a porous porcelain plate, where it was allowed

Table 12. *The effect of dithiocarbamyl compounds on the succinic oxidase activity in particulate fractions of yeast.* The main compartment in the Warburg flasks contained 2 ml phosphate buffer 0.02 *M*, pH 6.3, with Na-succinate to a concentration of 0.01 *M*. To the main compartment was further added 50 μ M zinc sulfate or 5 μ M cupric sulfate per liter, and the respective dithiocarbamyl compounds, as indicated in the table. MB was added in an amount of 0.1 ml 1 : 5000. The center cup contained 0.3 ml 5 % NaOH, and the side arm 0.2 ml particulate suspension in phosphate buffer. The side arm was tipped 15 min. after placing the flasks in the bath and readings taken during a 2 hour period from then on. The time from the start of the homogenization until the flasks were placed in the bath, was around 1 hour. The figures given in the table are μ l oxygen consumed in 2 hours, corrected for the oxygen uptake in flasks without succinate (the correction was usually very small), and the activity in per cent of that without the dithiocarbamyl compound.

Concentrations are given in μ moles per liter.

Experiment No. 1, with Na-DMDT:											
Na-DMDT, conc.:	0		1		5		10		100		
No heavy metal added,											
μl O ₂ uptake	47.0	100 %	47.5	101 %			42.5	90.5 %	12.0	25.5 %	
5 μM CuSO ₄ , μl O ₂ uptake	35.5	100 %	36.5	103 %	39.0	110 %	44.5	125 %			
50 μM ZnSO ₄ , μl O ₂ uptake	38.5	100 %	31.5	82 %			15.0	39 %	7.0	18 %	
Experiment No. 2, with Na-DMDT added:											
50 μM ZnSO ₄ , μl O ₂ uptake	54.0								7.0		
	50.0	100 %							7.0 10 %		
	48.0								3.0		
Experiment No. 3, with Na-DMDT and TMTD. The effect of MB.:											
Na-DMDT, conc.:	0		10		100						
50 μM ZnSO ₄ + MB, μl O ₂ uptake	41.5	100 %	9.0	22 %	10.0	24 %					
TMTD, conc.:	0		10		100						
No heavy metals,											
without MB, μl O ₂	53.5	100 %	13.0	24 %	2.5	4.5 %					
with MB μl O ₂	40.0	100 %	13.5	34 %	9.5	24 %					

to dry for 3 or 4 hours. The yeast was then scraped off, suspended in 10 ml 0.02 *M* phosphate buffer of pH 6.3 and transferred together with about 2 grams of sand (acid-washed, p.a.) to a Potter-Elvehjem homogenizer. This was placed in an ice bath, and allowed to cool before homogenization. The homogenization lasted about one minute, with the rotor at high speed. The contents were then transferred to a centrifuge tube of 15 ml capacity, and run for 10 minutes at 4000 r.p.m. in a small angle centrifuge. The cell-free homogenate could now be obtained by decantation. It was separated into a particulate (mitochondrial) and a soluble fraction by high-speed centrifugation for 20 minutes at approximately 15,000 *g*. As no refrigerated centrifuge was at hand, the centrifuge head was cooled by placing tubes containing ice in the holes not occupied by the homogenate. In this manner, the temperature of the homogenate during the centrifugation could be kept below 20–25° C. A MSE »Major» centrifuge was used in this experiment.

In some instances nitrogen determinations were made on the various fractions after the homogenization. Without giving detailed figures it may be mentioned that about 20—25 per cent of the cells were disrupted. The nitrogen content in the particulate fraction was about 15 per cent of that of the whole homogenate.

The first enzyme activity that was studied in homogenized cells was the succinic oxidase activity. The Warburg technique was employed. The particulate fraction was used, suspended in a phosphate buffer of pH 6.3. The main compartment of the flask contained the same phosphate buffer, with 0.01 *M* sodium succinate. The effect of Na-DMDT was tried in the phosphate buffer alone, and in the presence of zinc ions and cupric ions. The effect of TMTD was tried in phosphate buffer alone.

In absence of dithiocarbamyl compounds, the oxygen uptake was found to be linear for the first 2 hours or more. The oxygen uptake was of the order of magnitude of 25 μ l per hour, with 0.04—0.06 mg N in each flask. The Q_N^{air} value was thus of the order of magnitude of 500 μ l/mg per hour.

The results from the experiments are found in table 12. They show that Zn-DMDT and TMTD have a strong inhibitory action on the succinic oxidase system, whereas Na-DMDT has a somewhat smaller effect. The cupric complexes have not been found to be of any effect, the only result obtained when adding Na-DMDT being a counteraction of the effect of the heavy metal alone.

To see whether the inhibition occurred in the cytochrome oxidase system or the succinic dehydrogenase system, another experiment was carried out in which methylene blue (MB) was added. The inhibition (table 12) was found to be of the same order of magnitude in the presence of MB as without it, indicating that the main inhibition is caused in the succinic dehydrogenase system. These results should be compared with the effect of Zn-DMDT both on succinate oxidation by intact cells, where practically no inhibition was found, and on the glucose breakdown, where the degree of inhibition for the total oxidation was the same as for the anaerobic fermentation, and of a considerably lower order of magnitude than it is found here for the succinic oxidase system.

C. Alcohol Dehydrogenase (ADH)

Succinic dehydrogenase is considered to be an SH-enzyme (Schlenk, 1951b). As another SH-enzyme alcohol dehydrogenase, which could be expected to have a different behavior towards the dithiocarbamyl compounds, was chosen. Kjeldgaard (1949) has shown that the liver ADH was little affected by TETD

Table 13. *The effect of dithiocarbamyl compounds on the ADH activity in yeast homogenates, measured spectrophotometrically.* The Beckman cuvettes contained 2.8 ml glycine-NaOH buffer of pH 9.5, to which was added the various dithiocarbamyl compounds and zinc sulfate, as indicated in the table. 0.08 ml ethanol and 0.07 ml of a DPN solution (83.5 mg 60 % DPN in 5 ml glycine buffer) were also added. 0.03 ml yeast homogenate was added at zero time, and readings at 3400 Å taken after 1, 2 and 3 minutes. The figures in the table give the increase in extinction value at initial velocity during 3 minutes, multiplied by 100. The concentrations are given in μ moles per liter.

Na-DMDT, conc.	0	2	10	100
No heavy metal added, ADH activity	60.3			62.4
50 μ M ZnSO ₄ , ADH activity	20.4			
	19.2			
	19.0	19.0	18.4	17.0
TMTD, conc.	0	2	10	100
No heavy metal added, ADH activity	55.2	54.6	54.2	63.2
TMTM, conc.	0			200
No heavy metal added, ADH activity	55.2			55.5

The ADH activity of whole homogenates (prepared as under B) was followed spectrophotometrically by the reduction of added DPN in glycine buffer of pH 9.5.

Results are seen in table 13. It is noted that ADH is very sensitive towards zinc ions; 50 μ M zinc sulfate causes an inhibition of 65–70 per cent. Addition of Na-DMDT together with the zinc sulfate does not change the degree of inhibition. Na-DMDT and TMTD have no effect on the ADH activity.

D. The Acetate-Activating System

Jones, Black, Flynn and Lipmann (1953) have described the preparation and kinetic properties of an enzyme from yeast, catalysing the reaction:



As this reaction can for several reasons be believed to be the first step in acetate metabolism, a study was made of how it was affected by dithiocarbamyl compounds. For this purpose, partly cellular extracts (the first ammonium sulfate precipitates of Jones et al) and partly whole yeast was used. For an assay system was used trapping with hydroxylamine, and

Table 14. *The effect of Na-DMDT and Zn-DMDT on the enzymatic formation of acethydroxamic acid from sodium acetate and hydroxylamine.* — Assay system: See Jones, Black, Flynn and Lipman (1953). As enzyme preparation was used CO₂-frozen yeast. Na-DMDT was added to a concentration of 100 μ M, and ZnSO₄ to 50 μ M. The acethydroxamic acid formed after 1 hour at 30° C was measured as the ferric complex after centrifugation, in the Lumetron photocolormeter at 5000 Å (filter M 515).

Addition	+ ATP		+ GSH		None		+ ATP and GSH	
Zn + Na-DMDT	0.218;	0.211	0.306;	0.357	0.126;	0.120	0.377;	0.387
Zn alone	0.174;	0.165	0.343;	0.347	0.114;	0.122	0.329;	0.347
Na-DMDT	0.218;	0.197	0.343;	0.357	0.149;	0.201	0.409;	0.372
H ₂ O	0.197;	0.137	0.372;	0.338	0.149;	0.155	0.352;	0.383

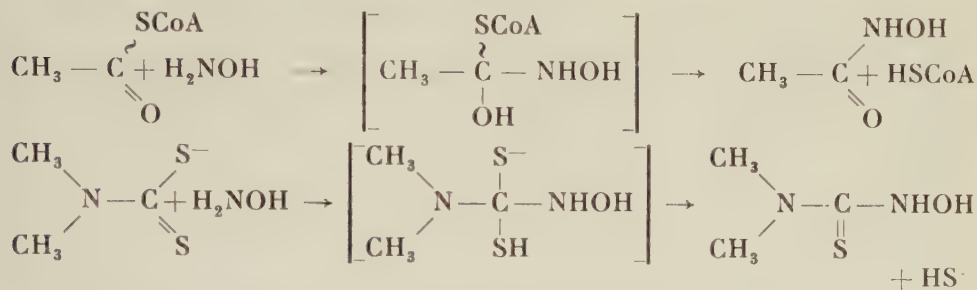
measurement of the ferric complex of acethydroxamic acid formed from acetyl coenzyme A.

The first experiments showed that when the complete assay system described by Jones et al, containing rather large amounts of GSH (0.01 M) was used, no effect of dithiocarbamyl compounds could be observed. Repeated experiments showed a strong activation effect by GSH even on crude extracts. As there was reason to believe that the dithiocarbamyl compounds would be counteracted by GSH (cf. the effects on animal aldehyde dehydrogenase), another experimental series was started, with ATP and GSH omitted from some of the samples. The results showed (table 14) quite surprisingly a slightly *stimulatory* effect of Na-DMDT or Zn-DMDT in this case. In the next experiment, fresh yeast was used, and since the yeast concentration had to be increased considerably above that used in Warburg or growth experiments in order to get readable results, the dithiocarbamate concentration was increased accordingly, up to 1000 μ M. Even in this case a slight stimulation occurred.

As a stimulation of the acetate-activating system did not seem compatible with the results found earlier in this work, an attempt was made to obtain a clearer picture of the cause of these results by carrying out an experiment in the constant temperature bath, where larger volumes of yeast could be handled, so that the process could be followed by removing samples at suitable time intervals for analysis. This time it was noted that when the stopper was removed for pipetting out a sample, *a strong odour of hydrogen sulfide emerged from the flasks that contained Na-DMDT and hydroxylamine.* When lead acetate paper was placed over the openings, the characteristic dark colour of lead sulfide appeared. This was very noteworthy, as sulfide has been used to reduce coenzyme A in acetate-activating experiments (Jones, Black, Flynn and Lipmann, 1953), and the stimulating effect of Na-DMDT

in the presence of hydroxylamine could now be explained as being caused by hydrogen sulfide formation.

The manner in which Na-DMDT and hydroxylamine could react to form hydrogen sulfide would be quite analogous to the reaction with activated acyl compounds:



Attempts to mix Na-DMDT and hydroxylamine failed, however, to demonstrate any formation of hydrogen sulfide. The question naturally arising then was whether the dithiocarbamic acid also needed some sort of activation before it could react with hydroxylamine. If this were so, it would lead to very interesting considerations regarding the mechanism of action of the dithiocarbamyl compounds. Experiments undertaken to study this are reported in the next section.

E. Preliminary Investigations Regarding the Formation of Hydrogen Sulfide from Dithiocarbamate in the Presence of Hydroxylamine and Yeast

It has not been possible in the present work to carry out more than preliminary studies on this highly interesting reaction, a closer understanding of which, it is believed, will reveal much of the chemical background for the mechanism of action of dithiocarbamyl compounds and perhaps also other sulfur-containing fungicides.

For these investigations it was necessary to find a sufficiently sensitive method for the determination of sulfide sulfur. After some less successful attempts with estimations based on the degree of coloration of lead acetate paper, and on the absorption in cadmium acetate, the method of Lindsay (Snell and Snell, 1936), based on the formation of methylene blue from p-amino-dimethylaniline, hydrogen sulfide and an oxidizing agent such as ferric chloride, was selected as the best one.

It was found that the determinations could be carried out directly in the substrate, preferably after precipitation of protein with TCA, as otherwise a strong absorption of methylene blue to the protein will cause low results. Neither Na-DMDT (quickly decomposed in the acid solution to carbon disulfide and dimethylamine) nor hydroxylamine interfered, if the spectrophotometric determination of the methylene blue formed was done with care. A very convenient technique was to

Table 15. *The formation of hydrogen sulfide from Na-DMDT and hydroxylamine in the presence of yeast.* Experiments in the constant temperature bath, with 200 ml of yeast suspension (10 grams fresh weight/liter) in phosphate buffer of pH 6.3, to which was added 500 μ M Na-DMDT and 0.1 M hydroxylamine hydrochloride, neutralized with KOH to pH 6.3. Slow aeration, hydrogen sulfide formation detected by placing lead acetate paper on the openings of the flasks.

Experiment No. 1.							
Flask No.	1	2	3	4	5	6	7
Yeast	fresh	fresh	fresh	fresh	boiled	boiled	no
NH ₂ OH	+	—	+	—	+	—	+
ZnSO ₄	+	+	—	—	+	+	+
H ₂ S form	++	—	+	—	—	—	—
Experiment No. 2.							
Flask No.	1	2	3	4	5	6	7
Yeast	fresh	fresh	boiled	boiled	boiled centrif.	fresh + NaAC	no
NH ₂ OH	added	added	added	added	added	added	added
ZnSO ₄	»	»	»	»	»	»	»
H ₂ S form	+++	+++	—	—	—	+	—

take up whole spectra in the Cary recording spectrophotometer with high scanning speed, and to use the difference in extinction between 6700 and 7200 Å for calculation of the amount of methylene blue present. In this manner, the »background colour» due to iron complexes, etc. interfered only to a negligible degree.

In the experiments with whole yeast, the constant temperature bath was used for incubation, with 200 ml in each flask. No aeration was used, but the magnetic stirring mechanism kept the yeast well suspended. At suitable time intervals, samples of 20 ml were pipetted into 50 ml centrifuge tubes containing 10 ml 10 % TCA. The tubes were stoppered at once, and centrifuged for 10 minutes, whereafter 10 ml from each was transferred to test tubes. To these test tubes was added 0.5 ml of a freshly prepared (not more than 24 hours old) solution of 0.04 grams p-amino-dimethylaniline in 100 ml 1:1 hydrochloric acid. The tubes were shaken, and 0.1 ml of a solution of ferric chloride in hydrochloric acid (10 per cent ferric chloride hexahydrate in 1:1 hydrochloric acid) added. The colour develops slowly, and the spectra were taken after 1–2 hours. It should be noted that if the system contains substances combining with the ferric ion or reducing it, more ferric chloride has to be added.

Each day, a known standard, prepared by adding sodium sulfide from a stock solution (prepared every few days by weighing out sodium sulfide and dissolving in distilled water) to the substrate-TCA mixture, was measured together with the unknown samples.

In the first of the experiments to be reported here, the qualitative demonstration of a thermolabile factor in yeast responsible for the formation of hydrogen sulfide was made (table 15).

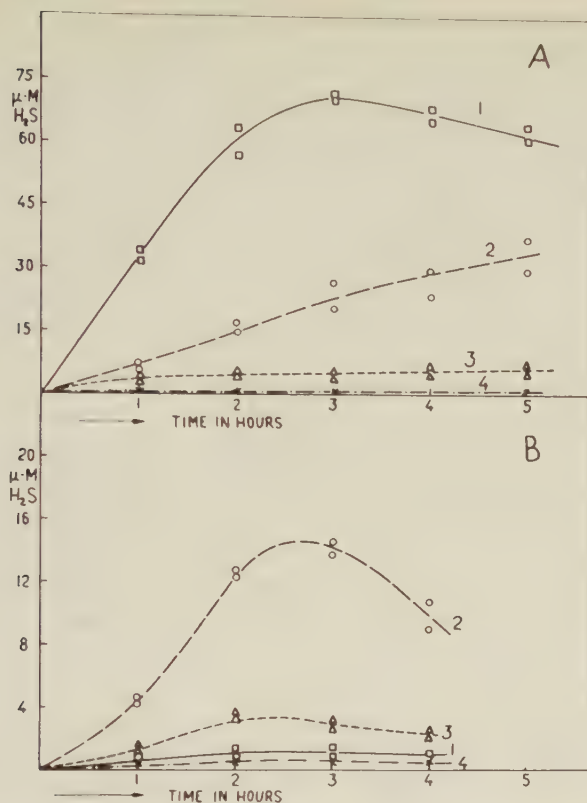


Figure 41. The rate of formation of hydrogen sulfide from yeast suspensions (10 grams fresh weight per liter) with hydroxylamine (0.1 M) and Na-DMDT added.

A, with 500 μM Na-DMDT. Curve 1, fresh yeast without zinc sulfate, curve 2, fresh yeast with 250 μM zinc sulfate. Curve 3, boiled yeast with 250 μM zinc sulfate. Curve 4, buffer system with 250 μM zinc sulfate, but with no yeast.

B, with 100 μM Na-DMDT. The curves have the same meaning as in A, the only difference being that zinc sulfate was added to a concentration of 50 μM .

Since from this experiment it seems as if zinc ions acted synergistic, the effect of zinc sulfate was studied at two concentration levels of Na-DMDT. In these experiments, the sulfide formation was also quantitatively determined during a period of several hours.

The results are shown in figure 41. It is seen that at low concentrations of Na-DMDT (100 μM), zinc is essential for a formation rate of hydrogen sulfide above the rate found in boiled yeast. At a Na-DMDT concentration of 500 μM , however, the hydrogen sulfide evolution is higher without zinc than with it. This latter result is probably explained by the low solubility of

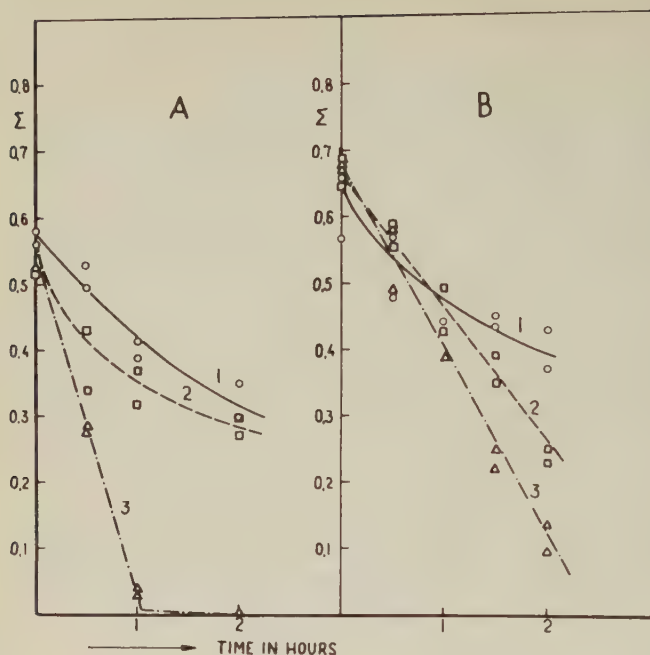


Figure 42. The stability of hydrogen sulfide in various systems.

A, with hydroxylamine (0.1 M) but without Na-DMDT.

B, without hydroxylamine, but with Na-DMDT (100 μ M). In both diagrams, curve 1 means fresh yeast (10 g/l), curve 2, boiled yeast, and curve 3, buffer system without yeast.

Zn-DMDT, and it would seem justified to conclude that at the »biological» concentration range of Na-DMDT, zinc ions are more or less essential for the reaction.

The curves in figure 41 show that sulfide must be very unstable in the substrate. In order to check this, and to find out whether the difference in hydrogen sulfide formation found between fresh and boiled yeasts, or in the buffer system alone, could be due merely to a difference in the stability of the sulfide, two experiments were done. In the first of these, sodium sulfide was added to the substrate from the start, and Na-DMDT was omitted. In the second, sodium sulfide was likewise added from the start, but this time hydroxylamine was omitted.

From figure 42 it can be seen that in the buffer system without Na-DMDT, sulfide oxidizes very rapidly, so that after one hour, virtually nothing is left. Both fresh and boiled yeast have a stabilizing action on sulfide, and these are of the same order of magnitude. The effect is probably due to a lowering of the redox potential of the solution. Na-DMDT is also seen to have a considerable effect on the stability of sulfide, probably also because of its low redox potential. It might be mentioned that in a preliminary experiment, the same effect was found with ascorbic acid.

The conclusion to be drawn from the curves in figure 42 must be that

Table 16. *The effect of the carbohydrate source on the hydrogen sulfide evolution in a yeast suspension containing 10 g/l in phosphate buffer of pH 6.3, with 500 μ M Na-DMDT and 0.1 M hydroxylamine. Hydrogen sulfide was measured quantitatively by the method of Lindsay. The results are given as the extinction values actually measured (the difference between the extinction values at 6700 and 7200 Å):*

Flask No.	1	2	3	4	5	6
C-source	Acetate	Acetate	Glucose	Glucose	None	None
H ₂ S formation after						
0 hour	0.01	0.01	0.01	0.02	0.02	0.02
1 »	0.31	0.26	0.26	0.27	0.27	0.27
2 hours	0.51	0.57	0.61	0.64	0.58	0.60
3 »	0.57	0.59	0.69	0.42	0.53	0.65

the difference in stability of sulfide under different conditions cannot explain the results presented in figure 41; accordingly, *a thermolabile factor in yeast must be responsible for the reaction leading to hydrogen sulfide formation from Na-DMDT and hydroxylamine.*

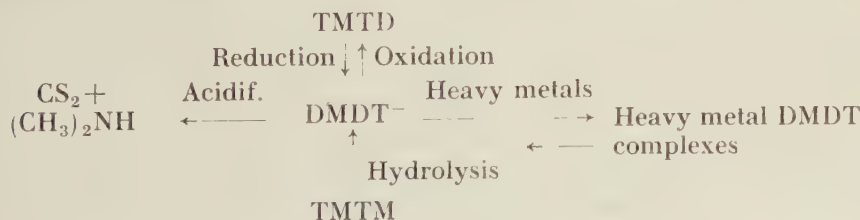
In the last experiment to be reported here, a study was made of whether addition of a carbon source to the yeast suspension would have any influence on hydrogen sulfide formation. No such influence was found (Table 16).

Some preliminary attempts have been made to extract the factor (presumably a protein) responsible for the reaction. These will not be reported in the present work, as there are many experimental difficulties to overcome before the assay system can be claimed to work satisfactorily, since it is based on the formation of hydrogen sulfide at a rate which by order of magnitude is the same as that with which it is normally oxidized in the substrate.

X. Discussion

A. The Chemical Reactions

The investigations performed in the first section of this work showed that there is no sharp and simple distinction between the different dimethyldithiocarbamyl compounds under investigation. They can be transformed into each other in the nutrient solution, partly by reversible, and partly by irreversible reactions:



In the nutrient solution, some sort of equilibrium will exist. If Na-DMDT is the substance added, with increasing concentrations will be formed: Cu-DMDT (1:1), Cu-DMDT (1:2), Zn-DMDT (1:1), Zn-DMDT (1:2), other heavy metal dithiocarbamates, and finally free dithiocarbamate ions. Part of the Na-DMDT will also be oxidized to TMTD, and part of it, depending on the pH of the solution, will hydrolyze to carbon disulfide and dimethylamine.

If Zn-DMDT is the compound added, it will partly dissociate, partly react with copper to form the two cupric complexes (the behaviour will probably depend on the relative concentrations of zinc and cupric ions), partly be oxidized, and partly hydrolyzed.

If Cu-DMDT is added, its low solubility will make it not readily available unless excess copper is present. In that case, probably the 1:1 complex will be formed.

It is pertinent also to discuss briefly the other heavy metal dithiocarbamates, in particular Fe-DMDT and Mn-DMDT.

The statement can be found in the literature (for instance, Horsfall, 1945) that it is the valence of the cation which determines the number of dithiocarbamyl groups in the heavy metal complexes. This is not the case. As stated by Koch (1949), the bonds between the metal and the sulfur atoms are of covalent character. It is therefore the *coordination number*, and not the valence of the metal that determines the number of dithiocarbamate groups. Fe^{+2} , Fe^{+3} and Mn^{+2} all have the coordination number of six, and an octahedral position of the ligands around the central atom. Since the dithiocarbamate ion acts as a bidentate, monovalent ligand, Fe (III)-DMDT must be electroneutral, while Fe (II)-DMDT and Mn (II)-DMDT have a negative charge, and can be expected to precipitate in the form of salts such as $\text{Fe} [\text{Fe}(\text{DMDT})_3]_2$ or $\text{Mn} [\text{Mn}(\text{DMDT})_3]_2$. The complexes are quite analogous to the betterknown cyanide complexes with iron or manganese.

It is evident that when, in dilute solutions, a partial dissociation of the complexes takes place, with the result that besides the saturated 1:3 complexes, 1:2 and 1:1 complexes are also found, the situation must be very complicated.

If TMTD is added to the nutrient solution, it will remain fairly stable until the conditions in the substrate are such that it becomes reduced. It will then form various metal dithiocarbamates, just as Na-DMDT does.

If TMTM is added to the nutrient solution, it will hydrolyse slowly, to form DMDT complexes and dimethylthiocarbamate.

It should be born in mind that the picture given here is perhaps still oversimplified, as it is assumed that the only reactions in which the dithiocarbamyl compounds take part are ordinary redox reactions, hydrolysis to carbon disulfide and amine, and complex-binding reactions. However, it is known that these compounds can undergo a number of other reactions, resulting in the formation of free sulfur, hydrogen sulfide, thiocyanate, and other more or less known compounds. But there is reason to believe that

these reactions proceed only to a slight extent under the conditions present in a nutrient solution, or in the protoplasm.

The behaviour of the other main group of dithiocarbamyl compounds, the bisdithiocarbamates, is very different.

B. *Experiments with Respiratory Systems*

In the oxidation of acetate, the acetate-activating system is the single enzymatic process known to precede the condensation reaction in which acetyl coenzyme A combines with oxalacetic acid and enters the Krebs cycle. It would therefore seem evident (since hypotheses assuming a different pathway in the oxidation of acetate than of glucose seem to have been abandoned) that, since the oxidation of glucose is much less affected than is the oxidation of acetate, it must be the acetate-activating system that is inhibited by the dithiocarbamyl compounds in intact yeast. The reaction mechanism of this system has recently been revealed, and found to consist of three sequences (Jones, Lipmann, Hilz and Lynen, 1953). In the first of these, an adenosine-phosphate-enzyme complex and pyrophosphate are formed from the enzyme and ATP. In the second, adenosinephosphate is substituted for coenzyme A in the enzyme complex. In the third reaction, acetyl coenzyme A and regenerated enzyme are formed.

The acetate oxidation has previously been found to be more or less selectively inhibited by three groups of inhibitors, namely fluoride, dinitrophenols and fluoroacetic (and iodoacetic) acid. As regards fluoroacetic acid, experiments by Kalnitsky and Guzman Barron (1947) on yeast, by Bartlett and Guzman Barron (1947) on animal tissues and by Black and Hutchens (1948) on yeast point in the direction of a competitive inhibition of the first step in the oxidative sequence. With other substrates like ethanol, glucose or pyruvic acid, acetate was found to accumulate in the presence of fluoroacetate. A similar action has been found by Stoppani and Deferrari (1950) in the case of iodoacetic acid or ethyl iodoacetate on acetate oxidation by yeast, at pH values around 4.8. At pH 7.3, no effect was shown by iodoacetic acid, whereas the effect of the ethyl ester was uninfluenced by pH. Stoppani concludes that the inhibition is competitive.

The effect of dinitrophenols on the oxidation of acetate by yeast has been reported by Stoppani (1949 a, b). A strong inhibition, practically specific for the acetate oxidation (Stoppani, 1949 b) was found at pH 4.8 or lower, whereas at pH 7.3, no inhibition took place.

Stoppani (1949 b) has also reported a strong inhibition of acetate oxidation by fluoride in yeast at low pH values, particularly if fluoride was added to starved yeast before acetate. Besides a pH effect on the inhibition,

incubation of the yeast with so-called acetate metabolism activators like ethanol, butanol, acetaldehyde, pyruvate or glucose counteracted the inhibition. Fitzgerald and Bernheim (1948) found at pH 6.0 a stimulation of the oxidation of acetate by fluoride in a strain (BCG) of *Mycobacterium*.

When comparing dithiocarbamyl compounds as a group of inhibitors with the other compounds that affect acetate oxidation, it would seem that they cannot be considered to act in a manner entirely similar to any of these. The inhibition with dithiocarbamates seems mainly non-competitive as regards acetate, but it is probably competitive as regards coenzyme A. The studies on the Pasteur mechanism demonstrated that the phosphorylation reactions were not uncoupled as with dinitrophenols. The action of fluoride on the acetate oxidation remains unclear. It should be noted that the acetate-activating assay system of Jones, Black, Flynn and Lipmann (1953) contains a considerable concentration of fluoride in order to inhibit the ATP-ase. This would mean that at least at pH 7.5, fluoride does not inhibit the acetate-activating system.

A discussion of a hypothetical manner in which the dithiocarbamyl compounds may act when inhibiting the acetate-activating system is given in section D of this chapter.

Besides the strong inhibition of the acetate oxidation, one noteworthy phenomenon observed during the respiratory experiments with intact cells was that the inhibition seems to concentrate on the *first* links in metabolic sequences. This was particularly pronounced with ethanol as the substrate. No inhibition of the carbon dioxide evolution on top of the inhibition of the oxygen uptake occurred then, indicating that the further oxidation of acetate in this case was unaffected. A similar behaviour was found with lactate as substrate. It is also remembered that the aerobic oxidation and the anaerobic fermentation of glucose were inhibited to the same degree, indicating that also in this case the sole inhibition must be ascribed to the first steps.

A further very important discovery is that when the cells were homogenized and the particulate fraction isolated, a strong inhibition of the succinic oxidase system appeared. It should be considered as definitely proven that the cytochrome system makes up the terminal link in the oxidative system, and that the succinic dehydrogenase is essential in the operation of the respiratory cycle. The only conclusion which it seems possible to draw, is that the dithiocarbamyl compounds are not capable of coming into contact with this enzyme system in intact cells, at least not in a state in which they can act as inhibitors.

The succinic oxidase system is known in animals and plants to be the key system in the mitochondria. It is very firmly bound to lipides, being inactivated when the lipides are hydrolyzed (Nygaard and Sumner, 1953).

It has been tentatively suggested that it should make up the cristae mitochondriales (Palade, 1953).

In yeast and other fungi, the information is more scanty, but recent investigations with ultra-rapid disintegration (Nossal, 1953, 1954 a, b) have revealed the existence of particulate material, resembling the mitochondria closely in dimensions and enzymic activity. The whole cyclophorase system has not yet, however, been demonstrated in particulate material from yeast. It should nevertheless be safe to conclude that in yeast cells, the succinoxidase system is located in mitochondriallike particles, which also contain a number of other mitochondrial functions. By further deduction, the conclusion is then reached that the dithiocarbamyl compounds cannot interfere with the other processes in the mitochondria, nor with processes in the nucleus. In fact, the dithiocarbamyl compounds cannot enter the interior of the intact cells to a degree which causes detectable inhibition, if lower concentrations than 1—200 μ moles per liter are used. Of course, these considerations are only valid for the earlier phases of the growth inhibition. At later stages, when the cells are dying, all sorts of reactions may occur.

A problem needing further consideration arises from the findings that on one hand, the first steps in the metabolic sequences are inhibited, and on the other hand, that it is only the processes located at the membrane that are affected. It might be asked, for instance, why acetate cannot enter the cell and become oxidized at the loci where the acetate formed from ethanol evidently is oxidized quite unaffectedly.

Possible explanations for this might be that: 1) the inhibition is connected with the permeation (this word is used to denote a passive process) through the cellular membrane, or, 2) the inhibition is connected with an active uptake mechanism, in the example mentioned, for instance, the process leading to acetyl coenzyme A from acetate, ATP and coenzyme A, (if this reaction is going on just inside the membrane, it will cause a strong difference in the chemical potential of acetate on both sides of the membrane, and thus induce permeation), or, 3) the oxidation from acetaldehyde goes directly to acetyl coenzyme A (inside the cell), whereas the acetate-activating enzyme is only located at the ectoplasm.

Of these possibilities, the second seems the most plausible, as on that basis, all the various findings may be satisfactorily explained. Thus, the observation that the oxidation of butyric acid, lactic acid, succinic acid, glucose or ethanol is less affected than the oxidation of acetic acid, seems to rule out the possibility that the structure of the membrane should have been altered so that acetic acid would not be able to permeate. On the other hand, if it is assumed that the third explanation would hold for acetic acid oxidation (there are virtually no observations supporting a supposition that the acetate-

activating system is located only at the ectoplasm), different explanations would have to be found for the inhibition which, although to a lesser degree, occurs with the oxidation of lactic acid and glucose.

If it is generally assumed that for acetate, lactate and glucose, reactions proceeding in or at the cellular membrane, by removing the various compounds and thereby creating potential gradients through the membrane, actively support the uptake of these compounds, and further, if it is assumed that these reactions may become inhibited by the DMDT compounds, an explanation is found which is satisfactory for the results reported in this work. The enzyme systems inhibited would then be: for glucose, the glucokinase system, for lactate, the lactic dehydrogenase system (which, contrary to other cytochromes, is very soluble), and for acetate, the acetate-activating system (the degree of inhibition is not the same in these three systems). The passive diffusion of these compounds into the cell would lead to a lower, unaffected rate of oxidation. Because of this, the degree of inhibition would be practically independent of the substrate concentration. The amount of substrate finally oxidized would be independent of the inhibitor concentration within reasonable limits.

It may further be concluded, that oxidizable substrates which cannot be attacked by an enzyme system just at the membrane would perhaps (at least in a number of cases) enter the cell at slower rates, and their oxidation would not be affected by the DMDT compounds. Examples of such substrates are succinate (attacked only by the mitochondria-bound succinic oxidase system), butyrate and probably L-tyrosine.

C. The Experiments with Growing Cells

The growth experiments demonstrated clearly the very happy choice of yeast as a test organism. Even though turbidity measurements are somewhat unspecific as a method of determining growth, they have furnished very clear and valuable information as regards the effect of dithiocarbamyl compounds on the growth process.

The observation that when Na-DMDT is added to actively growing yeast, the cell division stops immediately while the increase in turbidity still goes on for a while, points to blocking of a pathway essential for multiplication. It is, too, an argument against any explanation of the growth inhibition as being caused by lack of some essential compound, be it copper or something else. When the turbidity increases even though the cell number is constant, this must mean an increase in the absorbancy and/or light scattering of each cell. It seems quite obvious that this must mean an increase in the cellular content of each cell; that is, either an increase in volume or formation of a

more dense cellular material. Loveless, Spoerl and Weisman (1954) found, as previously mentioned, an increase in cellular volume up to 160 per cent of the controls, in yeast being growth-inhibited by Na-DEDT. This observation, given by them in a large compilation, has not by them been the subject of discussion, but it would seem to agree well with the findings reported in the present work.

The uptake experiments with labeled acetate show that the inhibition of acetate metabolism is not limited to the respiratory pathways in resting cells, but also affects the uptake during the early growth stages. In the early growth period, the assimilated acetate probably must pass in total directly into the anabolic processes, perhaps mainly towards lipide synthesis, since acetate is not easily oxidized during this period (Eaton and Klein, 1954). This would lead to the conclusion that the acetate metabolism must be inhibited at some early stage, probably before acetyl coenzyme A is formed, since otherwise different inhibition mechanisms would have to be proposed for each branch of the metabolic pathway.

When attempts to fractionate the radioactive compounds in growth-inhibited cells were made, a slight increase of isotope carbon in lipides was found, in comparison with that in normally grown cells. The difference was, however, not of a large order of magnitude, and it seems justified to ascribe it to a secondary process, following the inhibition of another energy-consuming pathway.

Very definite proofs seem to have been established for the fact that the protein metabolism at large is unaffected by dithiocarbamates. By the tracer experiments it was shown that the incorporation into proteins proceeded with the same relative rate in growth-inhibited as in normal cells. The experiments with low-N cells demonstrated that cells whose growth was completely stopped were even capable of building up a nitrogen reservoir of the same relative order of magnitude as ordinary growing cells.

The uptake of various nutrients (with the exception of glucose) in growth-inhibited cells has not been investigated. Some general conclusions might, however, be drawn on the basis of the present material.

The glucose utilization was found to parallel the growth very closely. This would mean that the «utilization coefficient» is the same in growth-inhibited as in normal cultures, when the calculation is based on glucose consumption.

From the experiments with low-N yeast it can be concluded that nitrogen uptake must proceed normally; hence ammonium ions must permeate unhampered. By chemical analogy, potassium permeation must also be expected to proceed unhampered.

The phosphorus metabolism connected with the synthesis of nucleic acids is, of course, of particular interest. It must be concluded that the growth

inhibition cannot be due to an inhibition of phosphate uptake, since it occurs so rapidly, and yeast cells are known to have a considerable phosphorus reservoir.

When discussing the mode of action of sulfur-containing fungicides, an interference with the sulfur metabolism might also be considered. However, the fact that parasitical fungi are even more sensitive than saprophytes (Klöpping, 1951), rules out the possibility of an effect on the sulfur assimilation at large. The plant parasites must be supposed to obtain their sulfur mainly in the form of S-containing amino acids which can be directly utilized by the fungi, and they should then be expected to be relatively insensitive to be dithiocarbamyl compounds, if these interfere only with the sulfur assimilation.

It should be stressed that this discussion has dealt with anabolic processes *at large*, that is, with the main pathways, without considering minor aberrations which would not be detectable when, for instance, measuring the radioactivity of the total lipid or total protein fractions. It is of course quite possible that the growth inhibition is due to the blocking of a reaction pathway, quantitatively negligible as compared to the main pathways. To rule out this possibility would be a virtually impossible task, as all the diverse compounds in the yeast cell would have to be analyzed with great accuracy, and with a knowledge of which differences were significant and which were not.

To the present author, work along this line seems unfruitful, and it is considered of greater importance to introduce cytological knowledge, and try to locate the physiological action *structurally*. Only after that would detailed biochemical discussions be of any value.

In section B of this discussion, it was concluded that the inhibitory action of the respiratory processes must be located at the cellular membrane. Although they are less definite, the growth studies lead to the same conclusion as regards the effect on anabolic processes. The internal anabolic processes proceed more or less unaffectedly in growth-inhibited cells, when seen in relation to the degree of growth, and the only decrease in metabolic activity was found in the processes leading to synthesis of cell wall material.

The observation of a sudden stop in cell division, but not in increase of cellular content, points in the direction of a blocking either of a process in the nucleus, or of a process connected with the growth of the cell wall or the cellular membrane. Mundkur (1954) has presented evidence showing that in yeast there is no distinct correlation between budding and division of the nucleus. The nucleus may start to divide before or after a bud has been formed. This seems to rule out the possibility of ascribing the inhibition of cell division to some inhibitory action on the nucleus, as then it would be expected that all cells containing nuclei which already had divided, would

divide once unhampered after addition of Na-DMDT, and one would get a decreasing rate of division for a short period.

The only well-defined metabolic pathway which has been found to be strongly inhibited by dithiocarbamyl compounds is the oxidation of acetate. It is not justified to consider this process as the cause of the growth inhibition. Acetate is not the natural substrate during growth. The acetate-activating mechanism must — according to present knowledge — rather be considered as a trapping mechanism with the purpose of catching the acetate which for some reason has been formed during metabolism, either by hydrolysis of acetyl coenzyme A, or by oxidation of acetaldehyde. It does not seem likely that loss of acetate for some time will be fatal to the cell. Therefore, inhibition of the acetate-activation system alone cannot be considered to be of vital importance.

More probable is the assumption that the acetate-oxidation inhibition is an indication of the inhibition of other enzyme systems, chemically similar to the system which forms the acetyl coenzyme A, and located in the cellular membrane or the ectoplasm.

The chemical constitution of the cell wall of the fungi is known to some extent. Regarding the cellular membrane, speculations can be found, but very little exact knowledge. As a whole our understanding of the metabolic processes taking place at the cellular membrane is very poor. It is somewhat surprising that this aspect of cellular metabolism has not been studied further, since a full understanding of the role of the cellular membrane and the ectoplasm certainly would clear up many puzzling and controversial points as regards the action of antibiotics as a whole.

At present, therefore, it is decidedly premature to present a detailed hypothesis explaining the physiological effects of the dithiocarbamyl compounds at the cellular membrane.

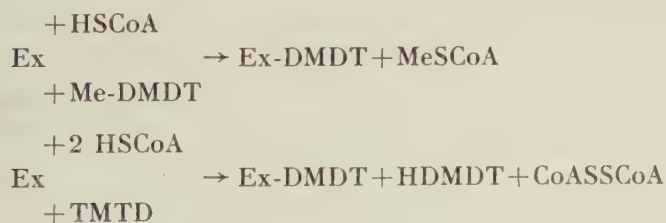
Although the effect of the dithiocarbamyl compounds on the acetate oxidation in itself has little to do with growth inhibition, it does give a good picture of the mechanism of action of the various dithiocarbamyl compounds. The results found regarding the inversion effect must thus probably hold true for the growth effect also, with the addition that superimposed on the enzymatic effect, a lack of copper at higher concentrations of Na-DMDT may occur, particularly when the inocula are small.

The findings of the van der Kerk group that heavy metals do not interfere with the effect of TMTD, should not be taken as a serious argument against the explanation of the inversion effect as, also with TMTD, being caused by Cu-DMDT (1 : 1) transforming into Cu-DMDT (1 : 2). It is simply not possible to carry out growth experiments without having in the nutrient solution the small amounts of cupric ions needed to form these complexes.

In other words, hydrogen sulfide formation would occur when hydroxylamine is added to the system. It is also seen that the active center in the enzyme molecule remains intact and, as the dithiocarbamate is destroyed by the reaction with hydroxylamine, a slight activation of the acetyl coenzyme A-forming system can be expected to occur because of the formation of hydrogen sulfide.

The reaction scheme presented above assumes a competition between the DMDT ion and coenzyme A for an active center in the protein molecule. This may be supported by the experiment in which it was found that incubation with calcium pantothenate reduced the sensitivity of the acetate oxidation mechanism to dithiocarbamate. Superficially, there is not much similarity between coenzyme A and Na-DMDT, but that does not necessarily rule out the possibility of a resemblance in the manner of chemical action. It should be remembered that the inhibitory effect of the dithiocarbamates decreases with increasing chain-length in the alkyl groups, the dimethyl compounds being by far the most active. This is in accordance with the conception of a reaction with an enzyme group, for which some definite steric conditions certainly must be set.

The findings that the 1 : 1 complexes of zinc and copper and TMTD are much more efficient inhibitors than the DMDT ion alone, can partly be explained by assuming that it is connected with the chemical stability of the compounds in solution, partly by assuming that these compounds are more easily transported through, or adsorbed on the membrane. A more interesting explanation can, however, also be presented, by assuming that these compounds may act in a manner rendering coenzyme A at least temporarily inactive, for example:



Now the enzyme which in intact cells is responsible for the formation of hydrogen sulfide cannot be assumed to be the acetate-activating enzyme — at least not this enzyme alone, if the reaction is connected with the growth inhibition. Other enzymes located in the cellular membrane or the ectoplasm, probably coenzyme A or other thiol-combining enzymes taking part in anabolic processes at this location must be supposed to be responsible for the reaction. The explanation presented above may hold for these enzymes

also, although less is known about the manner in which they react with coenzyme A.

The discussion would not be complete without a consideration of the »Antabuse effect». It would be very satisfying if a simple connection could be found between the specific inhibition of aldehyde dehydrogenases in animals, and the growth inhibition in fungi.

It has been proposed that TETD acts on aldehyde dehydrogenase by oxidizing the sulfhydryl groups of the enzyme to disulfides. When comparing the effect of TETD on alcohol dehydrogenase (ADH) and aldehyde dehydrogenase, it is seen that the explanation cannot be quite so simple as that. ADH and aldehyde dehydrogenase are both SH-enzymes, acting with DPN as a coenzyme, and even with a substrate, acetaldehyde, in common. TETD inhibits aldehyde dehydrogenase but not ADH. The difference in behaviour must lie in the particular reaction mechanism of each enzyme.

For animal ADH, Burton and Kaplan (1954) have proposed a reaction mechanism according to which ethanol combines with the DPN which is bound to the protein, whereafter ethanol is oxidized and DPN reduced via a transition state. According to this, DPN has a much more specific part in the reaction than merely to act as a second substrate for ADH.

The aldehyde dehydrogenases seem to act in an entirely different manner. With various preparations, several end products have been reported:

- | | |
|---|---|
| 1. $\text{CH}_3\text{CHO} \xrightarrow[\downarrow 2\text{H}]{\text{H}_2\text{O}} \text{CH}_3\text{COOH}$ | Yeast. (Black, 1951). Liver (Racker, 1949). |
| 2. $\text{CH}_3\text{CHO} \xrightarrow[\downarrow 2\text{H}]{\text{H}_3\text{PO}_4} \text{CH}_3\text{COOPO}(\text{OH})_2$ | Glyceraldehyde 3-phosphate dehydrogenase. (Harting, 1951, Harting and Chance, 1953). |
| 3. $\text{CH}_3\text{CHO} \xrightarrow[\downarrow 2\text{H}]{\text{CoA}} \text{CH}_3\text{CO S Co A}$ | Enzyme from <i>Clostridium cluyveri</i> . (Burton, 1952), rabbit muscle glyceraldehyde 3-phosphate dehydrogenase (Harting, 1951). |

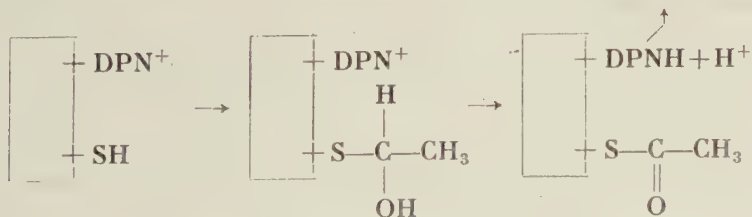
Singer and Kearney (1954) in discussing the mode of action of aldehyde dehydrogenases took as starting point the hypothesis presented by Racker (1951), in which a reaction mechanism similar to that in glyoxalase is assumed. Singer and Kearney tried to modify and extend this hypothesis by assuming that aldehyde dehydrogenase as an SH-enzyme combines with acetaldehyde in its hydrated form, whereafter two electrons are transferred

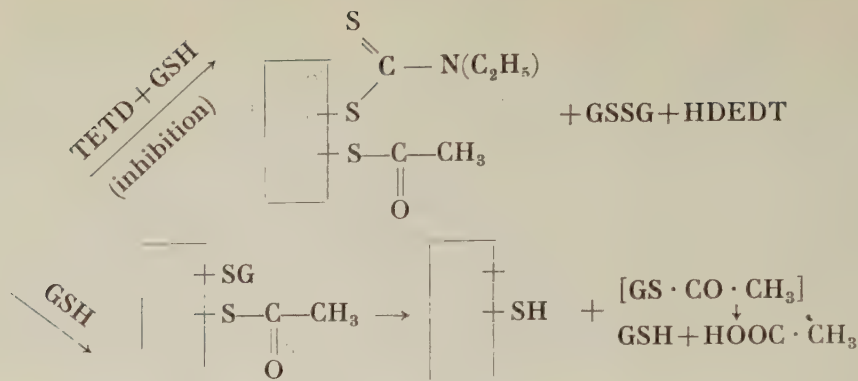
to the pyridine nucleotide. The thioester thus formed would be unstable if it were adjacent to a free amino group, and would liberate free acetic acid and the regenerated enzyme. This is the postulated mechanism for the aldehyde dehydrogenase isolated from yeast by Black (1951), which is shown not to require substrate amounts of cysteine or GSH (Singer and Kearney, 1954). The liver aldehyde dehydrogenase isolated from liver by Racker (1949) requires the addition of substantial amounts of SH compounds, such as cysteine. Singer and Kearney assume that with Racker's enzyme, an enzymatic or nonenzymatic transacetylation takes place, by which S-acetyl-cysteine and free enzyme are formed. S-acetyl-cysteine, in its turn, decomposes spontaneously.

In some cases, the thiol reacting with the acetyl-enzyme thioester may be coenzyme A. Then, acetyl coenzyme A would accumulate if no trans-acetylating systems were present, since it is a rather stable acyl mercaptan. As to whether the acetyl transfer from the enzyme to the thiol is enzymatic or not, Singer and Kearney assume that this may be determined by the affinity of the thiol for the dehydrogenase. The specific requirement of the dehydrogenase of *Clostridium cluyveri* for coenzyme A would suggest an enzymatic mechanism. The chemical energy of the reaction is lost as heat when acetic acid is formed, but is restored either in the thio-ester bond of acetyl coenzyme A or in acetyl phosphate.

From these considerations it is seen that a certain similarity exists between the aldehyde dehydrogenases and coenzyme A-combining enzymes, particularly transacetylases.

The effect of TETD on aldehyde dehydrogenase can be explained in a manner assuming reactions with this enzyme similar to the previously discussed reaction with the acetate-activating enzyme, viz., that TETD oxidizes SH in the protein and/or the RSH compound acting as prosthetic group, and in reduced form blocks the transacetylation mechanism in some manner. With Racker's enzyme, Graham (1951) found a competition between TETD and DPN, and a counteracting effect of GSH on the inhibition. This would lead to the following tentative scheme for the manner of action:





Other problems arise with regard to the strong inhibition of succinic dehydrogenase by dithiocarbamyl compounds. Keilin and Hartree (1940) studied the effect of Na-DEDT and TETD on this enzyme. They found an inhibition of Na-DEDT which could be ascribed to oxidation of the dithiocarbamate to TETD by cytochrome c. The inhibition by TETD was practically irreversible, and the activity was not restored even after washing, with or without precipitation of the enzyme. The activity was not restored by treatment with hydrogen sulfide, cyanide or dithionite. Only incubation for two hours with a high concentration of GSH produced a partial recovery of the activity. When added before the TETD, a much lower concentration of GSH was efficient in protecting the activity. It was further found that the alcohol treated preparations which had lost their ability to react with cytochrome c but which still could catalyze the reaction with methylene blue, were hardly affected by TETD.

Keilin and Hartree in discussing the manner in which TETD may act, assumed an oxidation of SH groups in the protein to disulfides. Since the dithiocarbamate formed by reduction of TETD during this oxidation can become reoxidized by cytochrome c TETD will behave like an electron mediator in this oxidation process. Besides, Keilin and Hartree stated that TETD has a general molecular configuration analogous to that of pyrophosphate. Pyrophosphate is considered to act as a competitive inhibitor by virtue of a molecular configuration which permits it to occupy the same active groupings in the dehydrogenase as succinic acid. The very low concentration at which TETD exerts a strong inhibitory effect indicated that the peculiar molecular structure makes it easily accessible to the same groups of the enzyme that react with the substrate.

It is interesting to compare the effect of a heavy metal (zinc) and dithio-

Table 17. *Per cent inhibition of ADH and succinic dehydrogenase by zinc sulfate (50 μ M), zinc sulfate plus Na-DMDT (50 μ M ZnSO_4 +100 μ M Na-DMDT), and TMTD. Data from tables 11 and 12.*

Addition	ADH	Succinic dehydrogenase
Zn^{2+}	66 %, 68 %, 68 %	18 %
Zn^{2+} + Na-DMDT	72 %	85 %
TMTD	0	96 %

carbamyl compounds on succinoxidase with that on alcohol dehydrogenase (table 17).

On succinic dehydrogenase, the heavy-metal dithiocarbamate complex has a much stronger action than the heavy metal alone; on ADH, the heavy metal acts as a strong inhibitor, but simultaneous addition of dithiocarbamate causes no difference in activity. TMTD is very active in the first case, inactive in the other. It seems to be quite clear that as regards succinic dehydrogenase, as well, the dithiocarbamate must react in a particular and specific manner, as these results do not seem compatible with a mere unspecific oxidation or blocking by heavy metals of thiol groups in the enzyme by the dithiocarbamyl compounds.

It is noted that the inhibition of the succinic oxidase system did not show the inversion effect in the presence of cupric sulfate. It was not considered of importance to check the findings by repeating the experiment, as only the qualitative inhibition of this system was considered to be of interest. A natural explanation would be that the lipide-phase in the particulate system has caused a shift in the equilibrium between the 1 : 1 and the 1 : 2 complex, since only the latter can be to any degree lipide-soluble.

E. Fungicidal Activity and Chemical Structure

It is now possible to discuss in somewhat greater detail the connection between fungicidal activity and chemical structure within the dithiocarbamyl group of fungicides.

The compounds investigated may be divided into three classes according to their effect on the acetate oxidation and probably on other enzyme systems vital for growth. The possibility of secondary reactions in the substrate, by which inactive compounds can be transformed into active, is not considered here.

From the results found in this work, the following two points are considered to be of primary importance in all cases:

1) Selection of a proper test organism. Not necessarily one which is of practical importance, but one which is suitable for the experimentations. Yeasts might in many cases be very advantageous. It is preferable to work out an hypothesis by means of a less representative organism which is easy to work with, and afterwards to try, by properly selected experiments, the validity of the hypothesis on other organisms, rather than to do all the time-consuming and difficult screening work with a number of organisms which are perhaps also difficult to work with.

2) A careful examination of the chemical reactions in the substrate in which the fungicide may take part. The substrate should also be as simple as possible; viz. liquid, and purely synthetic when this is possible. In many instances the atmosphere has to be considered as a part of the substrate. The results should be compared with the action curve of the fungicide.

After the determination of growth curves, together with the determination of the rapidity with which the growth inhibition appears after addition of the fungicide, and the effect on cell size, etc., a logical sequence of the physiological investigation might be to start with the glucose breakdown in intact cells. In connection with this, the results of this work show that the effect on the oxidation of acetate should be studied. All these screening experiments should be done as far as possible with the complete nutrient medium, since reaction products might be formed in the substrate which have the toxic action.

Next, the effect on the Pasteur mechanism, and on glucose consumption in growing cells will give information about the energy utilization and the utilization of the carbon source. The nitrogen assimilation might be separated from the growth process by using low-N cells, and studying the increase in nitrogenous constituents.

If the acetate oxidation is not too strongly inhibited, uptake experiments with acetate -C¹⁴ and fractionation of the cell contents, followed by comparison of the relative activity in the various fractions in growth-inhibited and control cultures, might give very valuable information about an eventual blocking of anabolic pathways.

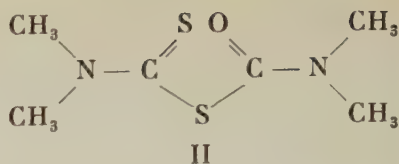
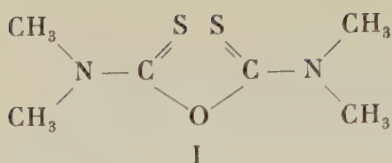
Comparisons of the effect of the fungicide on well-known reaction pathways in intact cells and in homogenates will perhaps in many cases give more reliable information about the localization of the fungicide in the cell, than will uptake experiments. It should be stressed that inhibitory experiments on enzymes in cell-free systems alone cannot furnish any evidence as to the mode of action.

When following a scheme like this, one should be able to discover the metabolic effects of a fungicide. It should then be possible to determine which type of reaction first is inhibited by the fungicide under investigation, and also, what is the chemical structure of the compound that really exerts the effect.

Summary

- 1) The chemical behaviour of the dithiocarbamyl compounds Na-DMDT, Zn-DMDT, Cu-DMDT, TMTD and TMTM (for explanation of the abbreviations see the preface) in a mineral salt nutrient solution has been investigated by spectrophotometric methods.

- 2) Values for the first and second stability constants for Zn-DMDT and Cu-DMDT have been estimated. Existence of a stable 1 : 1 complex between copper and dithiocarbamate was demonstrated spectrophotometrically.
- 3) When Cu-DMDT is formed in a solution containing proteins, weak bonds between the cupric atom and groups in the protein seem to be established, rendering Cu-DMDT un-extractable from such solutions.
- 4) It was shown that when added to a yeast suspension or a yeast extract, TMTD became reduced to dithiocarbamate.
- 5) Attempts to determine the rate of the uptake of dithiocarbamyl compounds by yeast demonstrated a very rapid equilibration. This points toward a surface adsorption rather than a permeation.
- 6) Respiratory studies with intact yeast cells revealed a strong inhibition of the acetate oxidation by heavy metal dithiocarbamate complexes with the exception of Cu-DMDT (1 : 2), and by TMTD, but not by Na-DMDT and TMTM.
- 7) The inhibition of the acetate oxidation was found to be practically independent of the acetate concentration. The amount of acetate finally oxidized with small additions of acetate was independent of the degree of inhibition, within practical limits.
- 8) Pretreatment of the yeast with pantothenate reduced the degree of inhibition of the acetate oxidation.
- 9) A study of the effect of varying concentrations of Na-DMDT on the acetate oxidation in the presence of low concentrations of cupric and zinc sulfate revealed an inversion effect. It is concluded that the inversion phenomenon shown by Na-DMDT on the growth can be explained in the same manner, namely:
 - a) At lower concentrations of Na-DMDT, inhibition due to the formation of Cu-DMDT (1 : 1)
 - b) Reversal of the inhibition by transforming the 1 : 1 complex to the 1 : 2 complex when increasing the concentration of Na-DMDT.
 - c) At further addition of Na-DMDT, new inhibition due to formation of Zn-DMDT, Mn-DMDT, Fe-DMDT, etc. At this stage, growth inhibition may even be caused by lack of copper, although this should be less general.
- 10) It was also demonstrated with TMTD and TMTM that the inversion phenomenon was caused by the system Cu-DMDT (1 : 1) \rightarrow Cu-DMDT (1 : 2). Whereas TMTD alone at higher concentrations caused inhibition of acetate oxidation, TMTM was wholly inactive at concentrations up to 200 μM .



F. Conclusions and Generalizations

The large number of observations of physiological character as regards the active dithiocarbamyl compounds made during this work, may be summarized in the following list:

- 1) No effect on other than the first links in metabolic sequences, when the metabolite was supplied externally.
- 2) No effect on the endogenous respiration.
- 3) Only a slight inhibition of the respiration with glucose as the substrate, but a strong inhibition with acetate.
- 4) Only a slight inhibition of the oxidation of butyric acid.
- 5) No effect on the oxidation of succinic acid in the intact cells, but a strong inhibition of the succinoxidase system in particulate fractions.
- 6) No inhibition of the Pasteur effect.
- 7) No effect on glucose consumption relative to growth, in growth-inhibited cells.
- 8) In growth-inhibited cells, the acetate-incorporation paralleled the rate of growth, and not the rate of oxygen consumption.
- 9) No effect on the nitrogen assimilation and protein synthesis when seen in relation to growth, in growth-inhibited cells.
- 10) A slight stimulation of lipide synthesis, and a distinct inhibition of the synthesis of cell wall material when seen in relation to growth, in growth-inhibited cells.
- 11) Uptake-curves of dithiocarbamyl compounds showed a very rapid establishment of equilibrium, indicating a surface adsorption, rather than a permeation.
- 12) When Na-DMDT was added to a growing culture, cell division stopped immediately, while the increase in cell contents went on for a while.
- 13) When yeast suspensions were incubated with Na-DMDT and hydroxylamine, a hydrogen sulfide formation occurred, and it was catalyzed by a thermolabile factor in yeast.

This is essentially a list of circumstantial evidence. It seems that all observations agree well if the conclusion is finally drawn — *as previously considered* — that the dithiocarbamyl compounds combine with particular groups in thiol-activated enzymes located at the cellular membrane or the

cytoplasmic layers just beneath (the ectoplasm), and act as poisons, by competing with the thiol working as prosthetic group for the enzyme. The most strongly inhibited enzymes must be those considered vital for the anabolic processes taking place at the membrane, but probably the compounds act more or less inhibitory on a rather large and diffuse group of enzymes at this location.

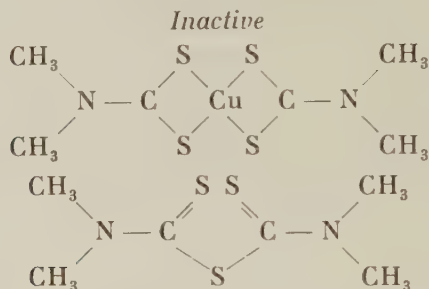
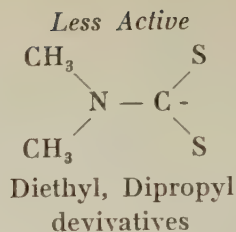
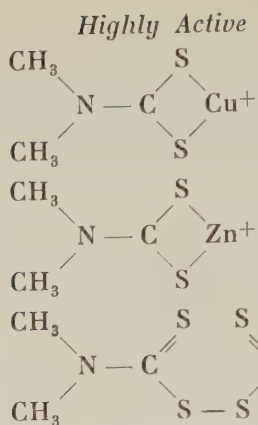
If the mode of action of the dithiocarbamyl compounds is such as that proposed above, it is easily understandable that the compounds may be very harmful to fungi and bacteria, and less toxic to plants and animals. Fungi differ from other plants in having chitin in their cell walls, and it may be that the difference in cell wall composition, being caused by a difference in cell membrane enzymes, would make them more sensitive towards the dithiocarbamyl compounds than other organisms. Organisms without chitin (primitive fungi and bacteria) in their cell walls are, however, also sensitive, and the inhibitory mechanism of action is also felt to be too general for such an explanation alone to be satisfactory.

According to the opinion of the present writer, anatomical differences may play a more important role than biochemical ones in this case. Bacteria and fungi are rapid-growing organisms with their cell walls freely exposed to the exterior, while higher plants and animals have only a body surface exposed, and this is often also protected by an impermeable layer.

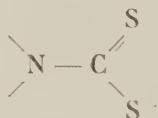
The conclusions reached from the experiments with *Saccharomyces cerevisiae*, and reported in this work, would need support from experiments carried out with other fungi before absolutely safe statements could be made as to the fungicidal mode of action in general. Such experiments have not been undertaken in the present work, as it is believed that when the relation between the enzymatic hydrogen sulfide evolution in the presence of dithiocarbamates and hydroxylamine and the fungicidal mechanism is better understood, only a set of quite simple experiments would be needed for a comprehensive statement of the results.

Is it possible to set up a general scheme for the evaluation of the manner of action of fungicidal compounds? This question arises as a natural result of the present report. It is important, because of the large amount of current work being done in fungicidal research, and the lack of system in the work that has been done.

The question might be answered with both yes and no. It is definitely possible to evaluate an experimental scheme which, with a minimum of research work, will tell which of the major groups of metabolic processes first becomes inhibited, and in many instances probably also, which enzyme system is most sensitive to the fungicide. On the other hand, by blindly following such a scheme, the research worker may in several cases be led completely astray, because a new fungicide might have properties which, when they are known, will alter the whole physiological view-point.



It is seen at once that a »biologically active group» of the type



cannot be responsible for the fungitoxicity, especially if this group plays a passive role within a larger molecule (as, for instance, in TMTM). Evidently, a requirement is that the dithiocarbamyl group must have an opportunity to react in some specific manner by its dithio acid radical.

On the basis of considerations concerning the biochemical mode of action of dithiocarbamyl compounds (cf. preceding section) a more detailed explanation of the difference in fungitoxicity of the various dithiocarbamyl compounds can be presented.

It is thus assumed that the dithiocarbamate ion competes with coenzyme A (and perhaps other thiols) for active centers in specific proteins. The steric conditions necessary for this make diethyl or dipropyl dithiocarbamates less active than dimethyldithiocarbamates. But an even more successful competition and, accordingly, a stronger inhibition is obtained if the thiol can

also be inactivated by the reaction, either by oxidation to a disulfide by TMTD, or by formation of heavy metal thiol complexes by Cu-DMDT (1 : 1) or Zn-DMDT.

Further, it is quite understandable that TMTM is inactive, since it does not have the opportunity either of combining directly with the protein or of reacting with the thiol in a manner which would enable it to combine with the protein and make the thiol inactive.

A point of practical importance is the necessity of distinguishing among the compounds (perhaps only a few in number) which are directly inhibitory, and those which have to undergo reactions in the substrate to become so. In previous literature on fungicidal research, this point is not seen to be stressed, although it is quite evident that several authors when discussing dosage-response curves, must have had it in mind. (A flat dosage-response curve would indicate that the compound is inactive in itself, and has to react before becoming fungitoxic.) In the evaluation of new fungicides this point is of importance, because on the basis of such a distinction, it should be possible to express the probability that a certain structure might have fungicidal activity, in physico-chemical terms, and thus to save a lot of random testing.

In the present case, compounds which are able to undergo reactions (hydrolysis, reduction, oxidation), which result finally in the formation of either heavy-metal DMDT complexes or TMTD, will be fungitoxic. Too little is at present known to state that all compounds with a dithiocarbamyl grouping which cannot undergo such reactions are inactive. Many of them certainly are. If the enzymatic mechanism of action were better understood, it would perhaps also be possible to make definite statements about members of this group.

These considerations can be illustrated by the behaviour of the compound »tetramethyl thiuram oxide« (I), which Klöpping (1951) has shown to be as fungitoxic as TMTM. This would not be compatible with the statement made above, that only compounds which could react with a dithio acid group, or compounds which could be transformed into such with a dithio acid group are fungitoxic. However, White (1954) has recently shown that the correct structure of this compound is not that of a thiuram oxide, but rather that of a dithiocarbamyl carbamyl sulfide (II). When the structure is of that type, a hydrolytic splitting of the compound would be expected to result in the formation of dithiocarbamate (besides thiocarbamate and carbamate). It is then quite understandable that the compound is fungitoxic, and in toxicity tests behaves like TMTM.

- 11) The inhibition of the various dialkyl derivatives of the acetate oxidation decreased in the order methyl > ethyl > propyl.
- 12) Addition of a chelating agent such as Versene counteracted strongly the inhibitory effect of Zn-DMDT on acetate oxidation. D,L-histidine had a similar, but less pronounced, action.
- 13) The effect of Zn-DMDT on the oxidation of ethanol, glucose, succinate, lactate, butyrate, and tyrosine, and on the anaerobic fermentation of glucose was compared with that on acetate oxidation. None of the reactions was inhibited to the same degree as the oxidation of acetate. The endogenous respiration was quite unaffected.
- 14) Under the experimental conditions used, no respiration was found by intact yeast with glutamate and D-alanine. With catechol and ascorbate, a rapid oxygen uptake occurred, but it was considered to be of a non-enzymatic character.
- 15) The effect of Na-DMDT, Zn-DMDT and TMTD on the Pasteur effect was studied. No inhibition was found.
- 16) In growing yeast, glucose consumption and acetate fixation went on at the same rate as the growth in growth-inhibited cultures, whereas the respiration was only to a small extent affected by the growth inhibition.
- 17) When the radioactivity was determined in the various fractions of yeast in the presence of labeled acetate, the same relative activity was found as in control samples in the fractions containing low molecular weight compounds, lipides (a slight increase in the growth-inhibited samples) and proteins, but a distinct decrease in the fraction containing mainly cell wall material.
- 18) In yeast with a low N content, the process of restoring the nitrogen reservoir was very little affected, even if the growth was completely arrested by Na-DMDT.
- 19) When Na-DMDT was added to a yeast suspension in full growth, the cell division stopped instantly, while the increase in turbidity still continued for a while.
- 20) With cell-free homogenates, a strong inhibition of the succinic oxidase system by Zn-DMDT and TMTD was found. Na-DMDT had less activity, while Cu-DMDT was inactive.
- 21) Dithiocarbamyl compounds did not affect the alcohol dehydrogenase activity in yeast homogenates.
- 22) In the presence of hydroxylamine, a thermolabile factor in yeast caused evolution of hydrogen sulfide from Na-DMDT. At lower concentrations of Na-DMDT, addition of zinc sulfate led to an increase in the reaction rate. Addition of carbon sources had no effect.

- 23) The results have been discussed. It is concluded that, as regards the mechanisms of action of the dithiocarbamyl compounds as fungicides, the various facts support an hypothesis which assumes that the compounds combine with particular groups at or in the cellular membrane, and in this location inhibit enzyme systems, probably by competing with coenzyme A or other thiols acting as prosthetic groups. The enzymes most strongly inhibited are assumed to be concerned with anabolic processes at the cellular membrane.

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References

1. Ambrus, J. L., Ambrus, C. M., & Harrison, J. W.: Effect of sodium diethyldithiocarbamate on thyroid activity, — *Am. J. Pharm.* 123: 129. 1951.
2. Andreva, T. F. & Zubkovich, L. E.: (Action of some enzymic poisons on photochemical action of isolated chloroplasts). — *Doklady Akad. Nauk. SSSR* 70: 525. 1950.
3. — — (Photochemical activity of isolated chloroplasts). — *Trudy Inst. Fiziol. Rastanii im K. A. Timiryazeva* 8, No. 1: 67. 1953.
4. Appleby, C. A. & Morton, K. K. Crystalline cytochrome b_2 and lactic dehydrogenase from yeast. — *Nature* 173: 749. 1954.
5. Arnon, D. I. & Whatley, F. R.: Metabolism of isolated cellular particles from photosynthetic tissues. I. Oxygen uptake and carbon dioxide-evolution in the dark. — *Physiol. Plant.* 7: 602. 1954.
6. Auerbach, F. & Bodenländer, P.: Zur Bestimmung von Glucose durch Oxidation mit Jod. — *Z. Angew. Chemie* 36: 602. 1923.
7. Bach, S. J., Dixon, M. & Keilin, D.: A new soluble component from yeast. — *Nature* 149: 21. 1942.
8. — — & Zervas, L. G.: Lactic dehydrogenase of yeast. — *Nature* 149: 48. 1942.
9. Balestrieri, A.: Ricerche intorno all'azione del T.T.D. su germi gram-positive e gram-negative. — *Atti Ist. Veneto Sci., Classe sci. mat. e nat.* 110: 25. 1952.
10. Barratt, R. W. & Horsfall, J. G.: Fungicidal action of metallic alkyl bisdithiocarbamates. — *Connecticut Agr. Expt. Sta. Bull* 508. 1947.
11. Bartlett, G. R. & Guzman Barron, E. S.: The effect of fluoroacetate on tissue metabolism. Its use for the study of the oxidative pathway of pyruvate metabolism. — *J. Biol. Chem.* 170: 67. 1947.
12. Beevers, H. & Gibbs, M.: Participation of the oxidative pathway in yeast respiration. — *Nature* 173: 640. 1954.

13. Beinert, H., Bock, R. M., Goldman, D. S., Green, D. E., Mahler, H. R., Mii, S., Stansley, P. G. & Wakit, S. J.: The reconstruction of the fatty acid oxidizing system of animal tissues. — *J. Am. Chem. Soc.* 75: 4111. 1953.
14. Bergem, N.: Contributions to the theories of vulcanisation, chiefly based on the T-50 test. — Askim Gummivarefabrik, Norway. 1948.
15. Black, S.: Yeast aldehyde dehydrogenase. — *Arch. Bioch. Biophys.* 34: 86. 1951.
16. — & Hutchens, J. O.: The oxidation of acetate by yeast in the presence of fluoracetate. *Arch. Bioch.* 17: 211. 1948.
17. Blackith, R. E.: Primary and secondary responses in biological assay. — *Nature* 161: 20. 1948.
18. Bloom, B. & De Witt Stetten Jr.: Pathways of glucose catabolism. — *J. Am. Chem. Soc.* 75: 5446. 1953.
19. Blouch, R., Payne, M. G. & Fults, J. L.: Free amino acids in sugar beet leaves altered by zinc dimethyldithiocarbamate. — *Bot. Gaz.* 114: 248. 1952.
20. Blumenthal, H. J., Lewis, K. P. & Weinhouse, S.: An estimation of glucose catabolism in yeast. — *J. Am. Chem. Soc.* 76: 6093. 1954.
21. Bodine, J. H. & Fitzgerald, L. R.: Effect of diethyldithiocarbamate on respiration of active and blocked embryonic cells. — *Proc. Soc. Exptl. Biol. Med.* 69: 442. 1948.
22. — — The formation of a complex between certain respiratory inhibitors and copper. — *J. Cellular Comp. Physiol.* 33: 215. 1949.
23. Boyer, P. D., Falcone, A. B. & Harrison, W. H.: Reversal and mechanism of oxidative phosphorylation. — *Nature* 174: 402. 1954.
24. v. Braun, J.: Zur Kenntnis der Thiuramdisulfide und Isothiuramdisulfide. I. — *Ber. Chem. Ges.* 35: 817. 1902.
25. — & Stechele, F.: Ueber Thiuramsulfide und die Einwirkung von cyanwasserstoffsäuren Salzen auf Disulfide. — *Ber. Chem. Ges.* 36: 2275. 1903.
26. Brieger, H. & Hodes, W. A.: Toxicity of dithiocarbamates and the hazard from exposure to these compounds. — *Proc. 9th Intern. Congr. Ind. Med. London* 1948: 598. 1949.
27. Burton, R. M.: Role of coenzyme A in acetaldehyde oxidation. — *Federation Proc.* 11: 193. 1952.
28. — & Kaplan, N. O.: A chemical reaction of hydroxylamine with diphosphopyridine nucleotide. — *J. Biol. Chem.* 211: 447. 1954.
29. Butler, G. W.: Ion uptake by young wheat plants. III. Phosphate absorption by excised roots. — *Physiol. Plant.* 6: 637. 1953.
30. Cantino, E. O. & Hyatt, M. T.: Further evidence for the role of the tricarboxylic acid cycle in morphogenesis in *Blastocladiella emersonii*. — *J. Bacteriol.* 66: 712. 1953.
31. Clarke, D. G., Baum, H., Stanley, B. L. & Hester, W. F.: Determination of dithiocarbamates. — *Anal. Chem.* 23: 1842. 1951.
32. Cox, C., Sisler, H. D. & Spurr, R. A.: Identity of gaseous toxicants from organic sulfur fungicides. — *Science* 114: 643. 1951.
33. Craig, D., Juve, A. E. & Davidson, W. L.: Tetramethylthiuram disulfide vulcanisation of rubber. I. Introduction and compounding. — *J. Polymer Sci.* 5: 709. 1950.
34. — Davidson, W. L., Juve, A. E. & Geib, I. J.: Tetramethyl thiuram disulfide vulcanisation of extracted rubber. II. Structure of radioactive tetramethylthiuram disulfide. — *J. Polymer Sci.* 6: 1. 1951.
35. Dale, B. G.: Catalase and esterase content of human liver obtained by needle biopsy. — *Am. J. Med. Sci.* 226: 42. 1953.

36. David, I.: Azione del tetraetilithiuramdisolforo (T.T.D.) sul consumo di ossigeno, sulle glicolisi anaerobia e sul potere deidrogenasico del lievito de pane. — *Acta Vitaminol.* 4: 23. 1951.
37. Davies, W. H. & Sexton, W. A.: Chemical constitution and fungistatic action of organic sulfur compounds. — *Bioch. J.* 40: 331. 1946.
38. Dawson, C. R. & Tarpley, W. B.: Copper oxidase. — In »The Enzymes», ed. Sumner & Myrbäck, Vol. II: 455. N.Y. 1951.
39. Delépine, M.: Composés sulfurés et azotés dérivés du sulfure de carbon (XII). Thio-sulfocarbamates métalliques. — *Bull. Soc. Chim.* 3: 643. 1908.
40. Dickens, F. D.: Anaerobic glycolysis, respiration and the Pasteur effect. — In »The Enzymes», ed. Sumner & Myrbäck, Vol. II: 624. N.Y. 1951.
41. Dickinson, D.: Determination of Fermate (ferric dimethyldithiocarbamate). — *Analyst* 71: 327. 1946.
42. Dimond, A. E., Heuberger, J. W. & Stoddard, E. M.: Role of the dosage-response curve in the evaluation of fungicides. — *Connecticut Agr. Exp. Sta. Bull.* 451: 635. 1941.
43. — & Horsfall, J. G.: Preventing the bacterial oxidation of rubber. *Science* 97: 144. 1943.
44. Dixon, M. & Zervas, L. G.: Lactic dehydrogenase and cytochrome. — *Nature* 143: 557. 1939.
45. Domar, G., Fredga, A. & Linderholm, H.: A method for quantitative determination of tetraethylthiuram disulfide (Antabuse, Abstinyl), and its reduced form, diethyldithiocarbamic acid, as found in excreta. — *Acta. Chem. Scand.* 3: 1441. 1949.
46. Eaton, N. R. & Klein, H. P.: The oxidation of glucose and acetate by *Saccharomyces cerevisiae*. — *J. Bacteriol.* 68: 110. 1954.
47. Edwards, T. P.: Effect of tetraethylthiuram disulfide on cellular respiration. — *Texas Repts. Biol. Med.* 7: 684. 1949.
48. Eldjarn, L.: The synthesis of tetraethylthiuram disulfide (Antabuse) labeled with radioactive sulfur. — *Acta Chem. Scand.* 3: 644. 1949.
49. — The metabolism of tetraethylthiuram disulfide (Antabuse, Aversan) in the rat, investigated by means of radioactive sulfur. — *Scand. J. Clin. Lab. Invest.* 2: 198. 1950 a.
50. — The metabolism of tetraethylthiuram disulfide (Antabuse, Aversan) in man, investigated by means of radioactive sulfur. — *Scand. J. Clin. Lab. Invest.* 2: 202. 1950 b.
51. Ferreira, P. C.: (Determination of bis(dialkylthiocarbamyl) disulfides). — *Arquiv. Biol.* 34: 103. 1950.
52. Fiess, H. A. & Klotz, I. M.: The thermodynamics of metallic protein combinations. Comparisons of copper complexes with natural proteins. — *J. Am. Chem. Soc.* 74: 887. 1952.
53. Fitzgerald, R. J. & Bernheim, F.: The effect of sodium fluoride on the metabolism of certain mycobacteria. — *J. Bacteriol.* 55: 677. 1948.
54. Freiman, D. G.: Use of an organic chelating agent in histochemical study of alkaline phosphatase action. — *Proc. Soc. Exptl. Biol. Med.* 84: 338. 1953.
55. Fruton, J. S. & Simmonds, S.: General biochemistry. — N.Y. — London 1953.
56. Fulfs, J. L., Payne, M. G., Gaskill, J. O., Hac, L. R. & Walker, A. C.: Glutamic acid in sugar beets increased by zinc dimethyldithiocarbamate. — *Botan. Gaz.* 113: 207. 1951.

57. Gilvarg, C. & Bloch, K.: The utilization of acetic acid for aminoacid synthesis in yeast. — *J. Biol. Chem.* 193: 339. 1951.
58. Goksøyr, J.: Reversal of the fungicidal effect of dithiocarbamyl compounds. — *Nature* 175: 820. 1955.
59. Goldsworthy, M. C., Green, E. L. & Smith, M. A.: Fungicidal and phytocidal properties of some metal dialkyldithiocarbamates. — *J. Agr. Res.* 66: 277. 1943.
60. Graham, W. D.: In vitro inhibition of liver aldehyde dehydrogenase by tetraethylthiuram disulfide. — *J. Pharm. Pharmacol.* 3: 160. 1951.
61. Gregg, E. C. & Tyler, W. P.: Polarography of the bis(diethylthiocarbamyl)disulfide-diethylthiocarbamate ion oxidation-reduction system. — *J. Am. Chem. Soc.* 72: 4561. 1950.
62. Guzman Barron, E. S. & Ghiretti, F.: The pathways of acetate oxidation. — *Bioch. Biophys. Acta.* 12: 239. 1953.
63. Hageloch, G. & Liebermeister, K.: Untersuchung über die keimhemmende Wirksamkeit einiger Stoffe mit der :NC(:S)-Gruppierung. — *Z. Naturforsch.* 6 b: 147. 1951.
64. Hald, J. & Jacobsen, E.: Formation of acetaldehyde in the organism after ingestion of Antabuse and ethanol. — *Acta Pharmacol. Toxicol.* 4: 285. 1948 a.
65. — — A drug sensitizing the organism to ethyl alcohol. — *Lancet* 255: 1001. 1948 b.
66. — — & Larsen, V.: The sensitizing effect of tetraethylthiuram disulfide (Antabuse) to alcohol. — *Acta Pharmacol. Toxicol.* 4: 285. 1948.
67. Harting, J.: Oxidation of acetaldehyde by glyceraldehyde-3-phosphate dehydrogenase of rabbit muscle. — *Federation Proc.* 10: 195. 1951.
68. — & Chance, B.: Discussion, Symposium on Chemistry and Function of Coenzyme A. — *Federation Proc.* 12: 714. 1953.
69. Horsfall, J. G.: Fungicides and Their Action. — *Mass. U.S.A.* 1945.
70. Houwink, A. L. & Kreger, D. R.: Observations on the cell wall of yeast. An electron microscope and X-ray diffraction study. — *Ant. van. Leeuwenhoek* 19: 1. 1953.
71. James, W. O. & Garton, N.: The use of sodium diethylthiocarbamate as a respiratory inhibitor. — *J. Exptl. Botany*, 3: 310. 1952.
72. Jeney, E., Zsolnai, T., Melles, Z. & Juhazy, P.: Untersuchung der bakteriostatischen Wirkung der Chelat-Komplex bildenden Verbindungen. — *Zentr. Bakteriöl. Parasitenkund. Abt. I. Orig.* 160: 677. 1954.
73. Johnston, C. D.: The in vitro reaction between tetraethylthiuram disulfide (Antabuse) and glutathione. — *Arch. Bioch. Biophys.* 44: 249. 1953.
74. — & Prickett, C. S.: Carbon disulfide from tetraethylthiuram disulfide by rat liver. — *Bioch. Biophys. Acta* 9: 219. 1952.
75. Jones, M. E., Black, S., Flynn, R. M. & Lipmann, F.: Acetyl coenzyme A synthesis through pyrophosphoryl split of adenosine triphosphate. — *Bioch. Biophys. Acta* 12: 141. 1953.
76. — Lipmann, F., Hiltz, H. & Lynen, F.: On the enzymatic mechanism of coenzyme A acetylation with adenosine triphosphate and acetate. — *J. Am. Chem. Soc.* 75: 3285. 1953.
77. Kalitsky, G. & Guzman Barron, E. S.: The effect of fluoracetate on the metabolism of yeast and bacteria. — *J. Biol. Chem.* 170: 83. 1947.
78. Keilin, D. & Hartree, E. F.: Succinic dehydrogenase-cytochrome system of cells. Intracellular respiratory system catalyzing aerobic oxidation of succinic acid. — *Proc. Roy. Soc.* 129 B: 277. 1940.

79. — & Mann, T.: Properties of laccase from the latex of laquer trees. — *Nature* 143: 23. 1939.
80. van der Kerk, G. J. M.: De fungicide werking van de dithiocarbaminaten en de bisdithiocarbaminaten. — *Chem. Weekblad* 49: 329. 1953.
81. — & Klöpping, H. L.: Investigations on organic fungicides. VII. Further considerations regarding the relations between chemical structure and antifungal action of dithiocarbamate and bisdithiocarbamate derivatives. — *Rec. Trav. Chim.* 71: 1179. 1952.
82. Kirchheim, D.: Toxicität und Wirkung einiger Thiuramdisulfid-Verbindungen auf Alkoholstoffwechsel. — *Arch. Exptl. Path. Pharmacol.* 214: 59. 1951.
83. Kjeldgaard, N. O.: Inhibition of aldehyde oxidase from liver by tetraethylthiuram disulfide (Antabuse). — *Acta Pharmacol. et Toxicol.* 5: 397. 1949.
84. Klöpping, H. L.: Chemical constitution and antifungal action of sulphur compounds. Utrecht 1951.
85. — & van der Kerk, G. J. M.: Organic fungicides. IV. Chemical constitution and fungistatic activity of dithiocarbamates, thiuram sulfides and structurally related compounds. — *Rec. Trav. Chim.* 70: 917. 1951.
86. Koch, H. P.: Absorption spectra and structure of organic sulfur compounds. III. Vulcanisation accelerators and related compounds. *J. Chem. Soc.* p. 401. 1949.
87. Krebs, H. A.: The intermediary stages in the biological oxidation of carbohydrate. — *Adv. Enzymology.* 3: 191. 1943.
88. Kress, K. E.: Spectrophotometric analysis of accelerator-rubber mixtures. — *Anal. Chem.* 23: 313. 1951.
89. Kubowitz, F.: Über die chemische Zusammensetzung der Kartoffeloxydase. — *Biochem. Z.* 292: 221. 1937.
90. Labbe, R. F., Thomas, R. C., Cheldelin, V. H., Christensen, B. E. & Wang, C. H.: Radioactive yeast fractions derived from carbon-14-labeled pyruvate and acetate. — *J. Biol. Chem.* 197: 655. 1952.
91. Lacoste, R. J., Earing, M. H. & Wiberley, S. E.: Colorimetric estimation of various metal derivatives of sodium diethyldithiocarbamate. — *Anal. Chem.* 23: 871. 1951.
92. Lardy, H. A. & Wellman, H.: Oxidative phosphorylations: Role of inorganic phosphate and acceptor systems in control of metabolic rates. — *J. Biol. Chem.* 195: 215. 1952.
93. Larsen, V., Jacobsen, E. & Hald, J.: Cause of the increased formation of acetaldehyde from alcohol in organisms treated with Antabuse. — *Intern. Congr. Bioch. Abstrs. of Commun.* 1st Congr. Cambridge, Engl. p. 6. 1949.
94. Leben, C. & Keitt, G. W.: Bioassay for tetramethylthiuram disulfide. — *Phytopath.* 40: 950. 1950.
95. Lees, H.: Effects of zinc and copper on soil nitrification. — *Biochem. J.* 42: 534. 1948.
96. Lehman, A. J.: Some toxicological reasons why certain chemicals may or may not be permitted as food additives. — *Assoc. Food & Drug Officials U.S. Quart. Bull.* 14: 82. 1950.
97. De Ley, J.: Over de fermenten van stichstofarme *Bacterium coli*. — *Gent* 1949.
98. Liebermeister, K.: Zum Wirkungsprinzip schwefelhaltiger tuberkulostatischer Chemotherapeutica. — *Z. Naturforsch.* 5 b: 79. 1950 a.
99. — Zum Wirkungsprinzip schwefelhaltiger tuberkulostatischer Chemotherapeutica. III. — *Z. Naturforsch.* 5 b: 254. 1950 b.
100. Linderholm, H. L. & Berg, K.: A method for the determination of tetraethylthiuram

- disulfide (Antabus, Abstinyl) and diethyldithiocarbamate in blood and urine. Some studies on the metabolism of tetraethylthiuram disulfide. — *Scand. J. Clin. & Lab. Investig.* 3: 96. 1951.
101. Lopatecki, L. E. & Newton, W.: The decomposition of dithiocarbamate fungicides with special reference to the volatile products. — *Can. J. Bot.* 30: 131. 1952.
 102. Loveless, L. E., Spoerl, E. & Weisman, T. H.: A survey of effects of chemicals on division and growth of yeast and *Escherichia coli*. — *J. Bacteriol.* 68: 637. 1954.
 103. Lowen, W. K.: Determination of dithiocarbamate residues on fruit crops. — *Anal. Chem.* 23: 1846. 1951.
 104. Ludwig, R. A., Thorn, G. D. & Miller, D. M.: The mechanism of fungicidal action of disodium ethylene bisdithiocarbamate. — *Can. J. Bot.* 32: 48. 1954.
 105. Lynen, F., Reichert, E. & Rueff, L.: Zum biologischen Abbau der Essigsäure VI. Aktivierter »Essigsäure«, ihre Isolierung aus Hefe und ihre chemische Natur. — *Ann.* 574: 1. 1951.
 106. Macheboef, M. A. & Viscontini, M.: Combinaisons entre protéides et cuivre. — *Compt. rend. (Paris)* 217: 305. 1943.
 107. Mahler, H. R.: Butyryl coenzyme A dehydrogenase, a cuproflavoprotein. — *J. Am. Chem. Soc.* 75: 3288. 1953.
 108. — Studies on the fatty acid oxidizing system of animal tissues. IV. The prosthetic group of butyryl coenzyme A dehydrogenase. — *J. Biol. Chem.* 206: 13. 1954.
 109. Malm, M.: Über die Permeabilität der Hefezellen und die von der permeierenden Stoffen, insbesondere Fluorwasserstoff, bedingten Plasmaveränderungen. — *Arkiv Kem. Min. Geol.* 25 A: 1. 1948.
 110. Manten, A., Klöpping, H. L. & van der Kerk, G. J. M.: Organic fungicides. I. Antimicrobial spectrum of the antifungal substance tetramethyl thiuram disulfide. — *Ant. van. Leeuwenhoek.* 16: 45. 1950.
 111. — — Investigations on organic fungicides. III. The influence of essential trace metals upon the fungitoxicity of tetramethylthiuram disulfide and 8-hydroxyquinoline. — *Ant. van. Leeuwenhoek.* 17: 58. 1951.
 112. Marsh, R. W.: Some applications of laboratory biological tests to the evaluation of fungicides. — *Ann. Appl. Biol.* 25: 583. 1938.
 113. Martell, A. E. & Calvin, M.: Chemistry of the metal chelate compounds. — N.Y. 1952.
 114. McCallan, S. E. A.: Dithiocarbamate fungicides. — *Agr. Chemicals* 1: 15. 1946.
 115. — Miller, L. P. & Weed, R. M.: Fungitoxicity of compounds possibly concerned in the mode of action of sulfur and Ferbam. — *Phytopath.* 42: 14. 1952.
 116. McQuillen, K. & Roberts, R. B.: The utilization of acetate for synthesis in *Escherichia coli*. — *J. Biol. Chem.* 207: 81. 1954.
 117. Meyerhof, O. & Fiala, S.: Pasteur effect in dead yeast. — *Bioch. Biophys. Acta* 6: 1. 1950.
 118. Miller, C. R. & Elson, W. O.: Dithiocarbamic acid derivatives. I. The relation of chemical structure to in vitro antibacterial and antifungal activity against human pathogens. — *J. Bacteriol.* 55: 47. 1949.
 119. Miller, L. P., McCallan, S. E. A. & Weed, P. E.: Rate of uptake and toxic dose on a spore weight basis of various fungicides. — *Contrib. Boyce Thompson Inst.* 17: 173. 1953.
 120. Montgomery, H. B. S. & Shaw, H.: Behaviour of thiuram sulfides, etc. in spore germination tests. — *Nature* 151: 333. 1943.
 121. Mundkur, B. D.: The nucleus of *Saccharomyces*: A cytological study of a frozen-dried polyploid series. — *J. Bacteriol.* 68: 514. 1954.

122. Mustard, M. J. & Lynch, S. J.: Effect of various factors upon the ascorbic acid content of some Florida-grown mangos. — *Florida Agr. Exptl. Sta., Bull.* No. 406; *Exptl. Sta. Record.* 93: 104. 1945.
123. Newman, H. W.: Antabuse and the metabolism of alcohol. — *Calif. Med.* 73: 137. 1950.
124. — Petzold, H. V.: The effect of tetramethylthiuram disulfide on the metabolism of ethyl alcohol. — *Quart. J. Studies Alc.* 12: 40. 1951.
125. Nossal, P. M.: A mechanical cell disintegrator. — *Austral. J. Exp. Biol.* 31: 583. 1953.
126. — Oxidative reactions in cell-free yeast extracts. — *Bioch. Biophys. Acta* 14: 154. 1954 a.
127. — Distribution of enzymes in cell-free yeast extracts. — *Biochem. J.* 57: 62. 1954 b.
128. — Incorporation of carboxy-¹⁴C-acetate into Krebs cycle acids in cell-free extracts. — *Bioch. Biophys. Acta* 15: 594. 1954 c.
129. Novelli, G. D. & Lipmann, F.: The involvement of coenzyme A in acetate oxidation in yeast. — *J. Biol. Chem.* 171: 833. 1947.
130. Nygaard, A. P. & Sumner, J. B.: D-glyceraldehyde-3-phosphate dehydrogenase; a comparison with liver aldehyde dehydrogenase. — *Arch. Bioch. Biophys.* 39: 119. 1952.
131. — The effect of Lecithinase A on the succinoxidase system. — *J. Biol. Chem.* 200: 723. 1953.
132. Palade, G. E.: The fine structure of mitochondria. An electron microscope study. — *J. Histochem. Cytochem.* 1: 188. 1953.
133. Palmer, H. C., Greenlee, R. W. & Baldwin, M. M.: Fungicidal action of sodium dialkyldithiocarbamates. — *Phytopath.* 42: 472. 1952.
134. Parker-Rhodes, A. F.: Mechanism of fungicidal action. V. Nonmetallic and sodium dithiocarbamic acid derivatives. — *Ann. Appl. Biol.* 30: 170. 1943.
134. Patterson, J. D.: Report on determination of dithiocarbamates. — *J. Assoc. Offic. Agr. Chemists* 33: 788. 1950.
135. Peyronal, G.: The coordinate hexavalency of amines of cupric halides. — *Gazz. chim. ital.* 71: 363. 1941.
136. Pfeiffer, P. & Pimmer, V.: Über Pyridin- und Chinolinhaltige Kupfersalze. — *Z. angew. Chemie* 48: 98. 1905.
137. van Raalte, M. H.: Het Effect van Metaal-ionen op de Fungistatische Werking van Natrium dimethyl Dithiocarbamaat. Mededel. Landbouwhogeschool, en Opzoekingsstas. — *Staat Gent*, 17: 163. 1952.
138. Racker, E.: Aldehyde dehydrogenase, a diphosphopyridine nucleotide-linked enzyme. — *J. Biol. Chem.* 177: 883. 1949.
139. — The mechanism of action of glyoxalase. — *J. Biol. Chem.* 190: 685. 1951.
140. Ramakrishnan, C. V. & Martin, S. M.: The »condensing enzyme» in *Aspergillus niger*. — *Chem. & Ind.* p. 160. 1954 a.
141. — — Inhibition of the »condensing enzyme» of *Aspergillus niger* by magnesium. — *Nature* 174: 230. 1954 b.
142. Richert, D. A., Vanderlinde, R. & Westerfeld, W. W.: The composition of rat liver xanthine oxidase and its inhibition by Antabuse. — *J. Biol. Chem.* 186: 261. 1950.
143. — & Westerfeld, W. W.: Xanthine oxidase in different species. — *Proc. Soc. Exptl. Biol. Med.* 76: 252. — 1951.
144. Robinson, F. A.: The Vitamin B complex. — London, 1951.

145. Roche, J.: Phosphatases. — In »The Enzymes», ed. Sumner & Myrbäck, Vol. I: 473. N.Y. 1950.
146. Rotchild, M. L. & MacVicar, R.: Studies on copper-containing oxidases in higher plants. — Federation Proc. 8: 245. 1949.
147. Sandell, E.: Fettoxidationsproblem inom farmasien. — Svensk Farm. Tidskr. 54: 473, 501, 525. 1950.
148. Schlenk, F.: Codehydrogenases I and II and apoenzymes. — In »The Enzymes», ed. Sumner & Myrbäck, Vol. II: 250. N.Y. 1951 a.
149. — Succinic dehydrogenase. — In »The Enzymes», ed. Sumner & Myrbäck, Vol. II: 316. N.Y. 1951 b.
150. Schrauffstätter, E.: Schwermetallkomplexbildung und antibakterielle Wirkung. — Z. Naturforsch. 5 b: 190. 1950.
151. Shibata, K., Benson, A. A. & Calvin, M.: The absorption spectra of suspensions of living microorganisms. — Bioch. Biophys. Acta 15: 461. 1954.
152. Sijpesteijn, A. K. & van der Kerk, G. M.: Investigations of organic fungicides. VI. Histidine as an antagonist of tetramethylthiuram disulfide (T.M.T.D.) and related compounds. — Ant. van Leeuwenhoek. 18: 83. 1952.
153. — — Investigations on organic fungicides. VIII. The biochemical mode of action of bisdithiocarbamates and diisothiocyanates. — Biochem. Biophys. Acta, 13: 545. 1954 a.
154. — — Investigations on organic fungicides. IX. The antagonistic action of certain imidazole derivatives and of α -keto acids on the fungitoxicity of dimethyldithiocarbamates. Bioch. Biophys. Acta 15: 69. 1954 b.
155. Singer, T. P. & Kearney, E. B.: The oxidizing enzymes. — In »The Proteins», ed. Neurath and Bailey, Vol. II A: 123. N.Y. 1954.
156. Sisler, H. D. & Cox, C. E.: Effects of tetramethylthiuram disulfide on metabolism of *Fusarium roseum*. — Am. J. Bot. 41: 338. 1954.
157. Snell, F. D. & Snell, C. T.: Colorimetric methods of analysis. — Vol. I: 593. N.Y. 1936.
158. Sorkin, E., Roth, W. & Erlenmeyer, H.: Über die Beeinflussung tuberkulostatischer Wirkungen durch Cu^{2+} . — Helv. Chem. Acta 35: 1736. 1952.
159. Steen, E.: Fotometriske studier av meningokokkens vekst i syntetiske medier. — Akademisk trykningsentral, Oslo. 1954.
160. Stoppani, A. O. M.: Effect of dinitrophenols on acetate oxidation by *Saccharomyces cerevisiae*. — Nature 164: 1096. 1949 a.
161. — Fluoride and dinitrophenol inhibition of acetate oxidation by *Saccharomyces cerevisiae*. — Intern. Congr. Biochem., Abstrs. of Commun. 1st Congr., Cambridge, Engl. 326. 1949 b.
162. — & Deferrari, J. O.: Accion del acido iodoacetico y del iodo-acetate de etilo sobre la oxidacion del acetato y del piruvato por *Saccharomyces cerevisiae*. — Anales asoc. quim. Argentina 38: 69. 1950.
163. Stotz, E., Harrer, C. J. & King, C. G.: A study of »ascorbic acid oxidase» in relation to copper. — J. Biol. Chem. 119: 511. 1937.
164. Tanford, C.: The effect of pH on the combination of serum albumin with metals. — J. Am. Chem. Soc. 74: 211. 1952.
165. Theorell, H.: Flavin-containing Enzymes. — In »The Enzymes», ed. Sumner & Myrbäck, Vol. II: 335. N.Y. 1951.
166. Tisdale, W. H. & Flenner, A. L.: Derivatives of dithiocarbamic acid as pesticides. — Ind. Eng. Chem. 34: 501. 1942.

167. — — Review of the derivatives of dithiocarbamic acid as fungicides. — Contrib. Boyce Thompson Inst. 16: 303. 1951.
168. Tollenaar, F. D.: The inhibition of oxidative defects in coldstored butter by some tetraalkylthiuram disulfides (preliminary report). — Netherlands Milk Dairy J. 5: 46. 1951.
169. Urba, R. & Burger, M.: [Coenzyme A and formation of citric acid by *Aspergillus niger* I.] — Chem. Listy 46: 175. 1952.
170. Vignais, P. & Vignais, P.: Sur l'oxydation de l'acétate par *Escherichia coli*. Variations en fonction de la croissance. Rôle inhibiteur de la streptomycine. — Compt. rend. (Paris) 238: 2127. 1954.
171. Viles, F. J.: Field determinations of carbon disulfide in air. — Ind. Hyg. Toxicol. 22: 188. 1940.
172. Virtanen, A. I.: On the adaptive formation of enzymes by micro-organisms. — Sv. Kem. Tidskr. 60: 23. 1948.
173. — Dependence of the enzyme activity of cells on their protein content. — Ann. Acad. Sci. Fenn. Ser. A, II Chem. No. 36: 1. 1949.
174. — & De Ley, J.: Enzyme activity and nitrogen content of bacterial cells. — Arch. Bioch. 16: 169. 1948.
175. — & Winkler, U.: Effect of decrease in the protein content of cells on the proteolytic enzyme system. — Acta Chem. Scand. 3: 272. 1949.
176. Wang, C. H., Labbe, R. F., Christensen, B. E. & Cheldelin, V. H.: Utilization of carbon 14-labeled pyruvate and acetate by yeast. — J. Biol. Chem. 197: 645. 1952.
177. — Thomas, R. C., Cheldelin, V. H. & Christensen, B. E.: Conversion of acetate and pyruvate to aspartic acid in yeast. — J. Biol. Chem. 197: 663. 1952.
178. Wase, A. W. & Christensen, J.: Antithyroid action of tetraethylthiuram disulfide. — J. Biol. Chem. 211: 75. 1954.
179. Weed, R. M., McCallan, G. E. A. & Miller, L. P.: Factors associated with the fungitoxicity of Ferbam and Nabam. — Contrib. Boyce Thompson Inst. 17: 299. 1953.
180. White, R. W.: The structure of the thiuram oxides. — Can. J. Chem. 32: 867. 1954.
181. Yamada, T. & Mizuno, K.: Crystal structure of accelerators of the thiuram series. A preliminary report. — J. Soc. Rubber Ind. Japan 15: 436. 1941 a.
182. — — Crystal structure of some thiuram-type accelerators. — J. Soc. Chem. Ind. Japan, 44: 708. 1941 b.

Notes on Temperature Measurements Indicative of Special Organization in Arctic and Subarctic Plants for Utilization of Radiated Heat from the Sun

By

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The warming of plants and poikilothermic animals by solar radiation has long been known (Rücker, 1933, 1934, Wigglesworth, 1939, Gunn, 1942). The possible existence of special organizational features of plants and animals in arctic and subarctic regions facilitating the utilization of solar energy for warming purposes seems to have been disregarded.

Arctic and subarctic regions have a longer winter than summer. The length of the season when the air is above freezing decreases with increasing latitude. The short summer, however, has long days which by prolonging diurnal activity accelerate growth and development. Cold and the shortness of the season, on the other hand, limit the number of species which can complete their reproduction within the short summer and thereby succeed in surviving in this habitat. Any peculiarity of an organism making it able to lengthen its season or accelerate its growth by solar radiation would therefore be of considerable importance.

It is well known that early in spring, when the air is still many degrees below freezing, solar radiation can warm dark objects above the temperature of the surrounding air. This fact has been realized by farming populations living in northern climates and is illustrated by their practice of the earlier clearing of their fields from snow for spring cultivation by spreading dirt or ashes in order to increase the absorption of heat from the sun.

The swelling of buds on the trees is an early sign of their warming by the sun to temperatures above freezing. The buds of the pussywillow bursting into catkins

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is one of the earliest signs of spring growth. The hairy covering of this *Salix* catkin is characteristic of the genus. Species such as *Salix polaris* which barely rises above the lichens and mosses among which it grows, are among the plants that grow farthest north. The early appearance of pussywillows in spring, often before the air warms above freezing, indicates that the sun must heat them up to temperatures sufficiently high for development. It is, therefore, of interest to examine the heating of willow catkins by radiation.

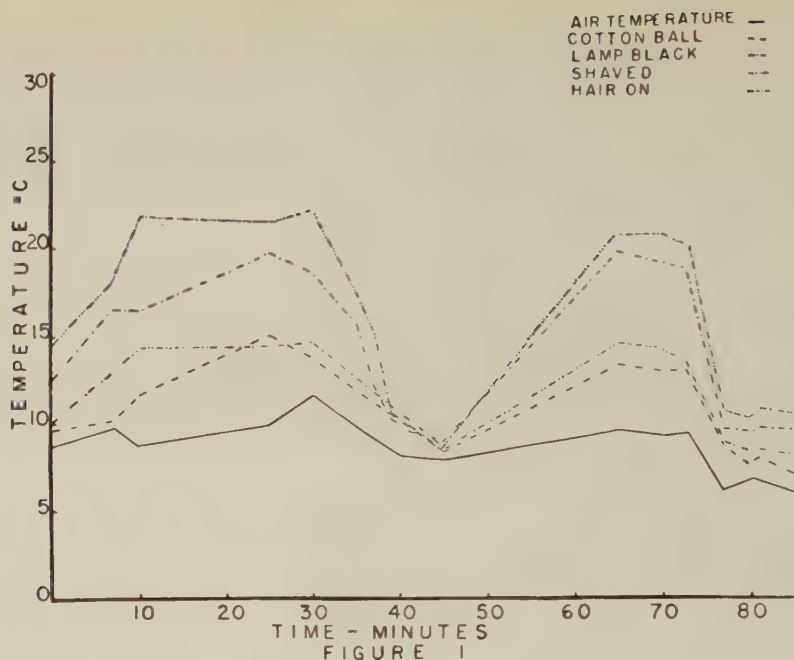
Experimental results

Fine needle thermocouples were placed in the center of catkins in the bright noon sun at Anchorage, Alaska. At air temperatures around freezing catkins became up to 15—25° C warmer than the surrounding air. This surprisingly high efficiency in heat absorption was unexpected considering the light color of the pussywillow, which would be expected to facilitate reflection of the sunlight rather than its absorption.

The following experiment was therefore planned to investigate the situation further. One catkin was left in its normal condition, care being taken not to disturb the layers of hair when introducing the thermocouple. A second was blackened with lamp black in order to eliminate reflection from the hairs and facilitate absorption. From a third catkin the hairs were clipped off down to the black undercoat in order to determine the importance of the hairs in preventing cooling by conduction and convection. A fourth thermocouple was placed inside a ball of cotton about the same size and shape as a catkin in order to get a comparison between the pussywillow and an object with about the same color and size. The air temperature was recorded with a bare needle thermocouple. All objects were placed side by side in the sun and their temperatures were recorded. Figure 1 shows the temperature curves obtained from the readings, the center dip of the curves being caused by shading which temporarily cut off the sunlight. From the graph it can be seen that the pussywillow left in its natural state showed the highest temperature increase above the surrounding air. Next to this came the catkin with the lamp black, and then followed in sequence the clipped one and the cotton ball.

Discussion

It had been anticipated that the darkened catkin would warm above the natural one with its shiny hair. The measurements show, however, that this was not the case. This leads to the hypothesis that the shiny hair on the pussywillow in one way or another facilitates heating by the sun rays. Before discussing the physical explanation of this hypothesis the structure of the pussywillow might be worth recapitulating. The soft outer layer consists of shiny transparent hairs. Underneath is a layer almost black in color, con-



sisting of the dark scales surrounding the flower buds. Then follow the layer of flower buds and the center core consisting of a more or less homogeneous parenchymatic tissue. With this structure in mind a physical explanation may be proposed.

The radiation from the sun with its greatest intensity in the yellow part of the spectrum will, upon hitting the hair of the pussywillow, be partly reflected, partly absorbed, and will partly penetrate the layer of hairs. The part of the rays that penetrates the hairs would be absorbed in the dark undercoat which would cause the center of the catkin to warm. This energy in the form of heat could again be partly lost either by radiation or by conduction to the surrounding air without appreciably increasing the temperature of the pussywillow. Such heat loss is probably reduced by the formation of a dead air space around the center of the catkin, decreasing convection and thereby heat loss due to conduction and evaporation. This explains the increased absorption of heat by the blackened pussywillow as compared to the clipped one. The greater heating of the naturally colored catkin can not be fully explained in that way. The most likely explanation is that the shiny hairs which let through a great deal of sunlight to be absorbed by the dark center of the catkin are opaque to the long waved radiation directed from the center outward. The pussywillow thus forms a »heat trap» working on

the same physical principle as a greenhouse or the type of oyster pools in which a floating layer of fresh water above the salt water seals it off from heat loss to the atmosphere.

The advantage of such a physical system for plants living in arctic and subarctic regions is obvious. It is therefore not surprising to find that other arctic plants and animals possess similar arrangements. As an example can be mentioned the arctic cottongrass, *Eriophorum*, which has a dark colored spikelet covered with shiny bracts. This plant, incidentally, is the next earliest plant to flower in the spring after the willows. The writer observed on one occasion in Anaktuvuk Pass in arctic Alaska that the spikelets of this plant were yellow with pollen on the side turned toward the sun, while the opposite side was still black and undeveloped. The air temperature at the time of observation was well below freezing.

Woolly bear caterpillars (*Arctiidae*), which are often seen crawling over the snow in spring, have long shiny black hairs emerging through a finer black under-layer. In bright sunshine one of these caterpillars was, by means of thermocouple measurements, found as much as 15° C warmer than the air over the snow upon which it had been crawling.

These are examples of physical arrangements by means of which some kinds of animals and plants warm themselves by the retention of solar radiation. These devices, which secure heat, can in effect prolong the arctic season for the animals and plants which bear them, for they may thereby start a sufficient metabolism for the essential cyclic activity of summer earlier, and by proceeding at a faster rate be able to complete the necessary sequences for reproduction within the short arctic summer. In this manner the geographical range of these forms may through special devices for the retention of radiant heat be extended considerably north of the limits indicated by the air temperature alone.

It is possible that interesting correlations may be found between the geographical distribution of an animal or plant and the amount of radiated solar energy available in a certain area. Routine observations of solar radiation, if included in meteorological records, would in this case be of considerable value to biologists working on problems of distribution.

References

1. Gunn, D. L.: Body temperature in poikilothermal animals. — *Biol. Rev.* 17: 293. 1942.
2. Rücker, F.: Die Farben der Insecten und ihre Bedeutung für den Wärmehaushalt. — *Pflüg. Arch. ges. Physiol.* 231: 729. 1933.
3. — Über die ultrarote Reflexion tierischer Körperoberflächen. — *Ztschr. vergl. Physiol.* 21: 275. 1934.
4. Wigglesworth, V. B.: *Principles of Insect Physiology*. — London, Methuen. pp. 359—372. 1939.

Variations in the Silica Content of Diatoms

By

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Studies on the silica content of diatoms have previously been published by Einsele & Grim 1938 and Lund 1950. In the paper by the former a great number of determinations of the silica content in diatoms were carried out. They examined 15 different species from plankton of different lakes in Austria and showed that the content of silica is very different in cells of the different species; but they also showed that the content of the cells of a single species varies a great deal.

The problem about the variations of silica content in cells of a single species is discussed by them on pp. 564—581 and it is pointed out that the content varies with the number of cells in the population. When the number of cells is high the content of silica is lower than in populations with fewer individuals. The silica content is inversely proportional to the number of cells.

Lund showed the same in his paper. In his Table 3 the number of cells per ml and the corresponding values of SiO_2 per 10^6 cells are arranged for various stages in the growth of a culture of *Asterionella formosa*. The number of cells in the culture increased from 22 cells to 249,000 cells per ml. Correspondingly the silica content decreased from 0.142 to 0.120 mg SiO_2 per 10^6 cells.

Both Einsele & Grim and Lund thought that the variations in the silica content are caused by the different growth rates. Thus Lund writes (p. 23): »The faster the growth rate the less the mean weight of silica per unit of population is likely to be, since cells in the process of division will not have fully silicified walls.»

However, the different growth rate cannot be the real explanation of the

problem. In a culture with a constant growth rate there is a higher average content of silica in the cells in the beginning of the culture period when few cells are present per unit than later with many cells.

Experiments

In the growth experiments with two epiphytic species, *Nitzschia palea* and *Bacillaria paradoxa* (Jørgensen 1953), the relation between the growth, expressed as increase in chlorophyl, and the silica uptake was shown. In some of the cultures the number of the cells was counted and the silica content in the cells calculated. In figures 1 and 2 the ratio between the silica content expressed as Si μg per 10^6 cells and the number of cells in the two experiments is shown. It is evident that there is an inverse ratio between the number of cells and the silica content. This cannot be due to differences in the growth rate. In the single experiment with the same initial silica concentration the growth rate is constant during the experiment until the growth stop on account of the silica concentration has reached a minimum value (see figure 3, the experiment in Table 1 with 6 mg Si per l.). It is also evident that the silica content in the cells is highest in the experiments with the highest initial silica concentration when the same number of cells is considered.

The same result as reported here for the epiphytic diatoms was obtained in experiments with a planktonic species *Thalassiosira nana* Lohmann. In a previous paper (l. c.) this species was wrongly referred to as *Th. fluviatilis* Hustedt. In Table 1 corresponding values of the number of cells and Si μg per 10^6 cells are stated for two experiments with the initial Si-concentration of respectively 2 mg Si per l and 6 mg Si per l. The table shows similar ratios as reported for the epiphytic species.

On the basis of these experiments where the Si-concentration decreases with increasing number of cells, it seems reasonable to conclude that the decrease in silica content in the diatoms is due to the decreasing Si-concentration. That this conclusion cannot be right is shown in the following experiments.

Table 1. *Experiments with Thalassiosira nana.*

2 mg Si per l	Number of cells per ml. Si μg per 10^6 cells	2,300 430	9,200 143	22,500 93
6 mg Si per l	Number of cells per ml. Si μg per 10^6 cells	42,800 132	63,300 97	69,200 89

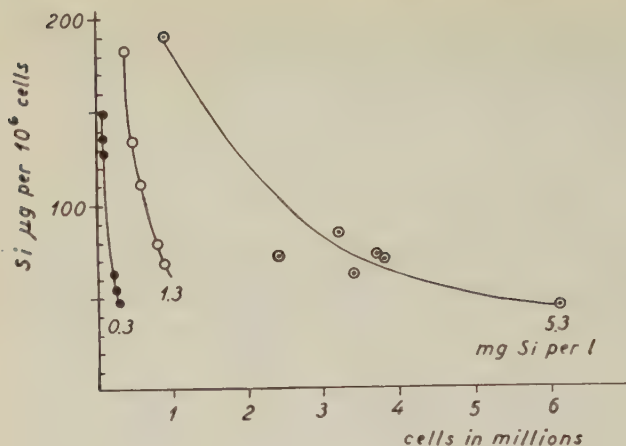


Figure 1. The ratio between the silica content expressed as $\mu\text{g Si per } 10^6 \text{ cells}$ and the number of cells of *Nitzschia palea* in experiments with the initial silicon concentration of 0.3, 1.3, and 5.3 mg Si per l.

Experiments with flowing culture solutions. In the above-mentioned experiments the Si-concentration decreased during the experiment and therefore it is difficult to examine exactly how the Si-concentration influences the silica content of the cells. So some experiments with flowing culture solutions were carried out. During these experiments the Si-concentration was approximately constant. In such experiments it is only possible to work with epiphytic species which are well fixed to the culture containers. This is the case with *Nitzschia palea* in containers of plastics.

The culture solution was the same modification of Rodhe VIII as used in previous experiments with *Nitzschia palea* (Jørgensen 1952). The diatoms in some experiments were cultivated in containers of polystyrene in others in bottles of polyethylene. The diatoms were inoculated and cultivated in these containers in the ordinary way for some days. The current of culture solution was not started before distinct colonies of diatoms were visible.

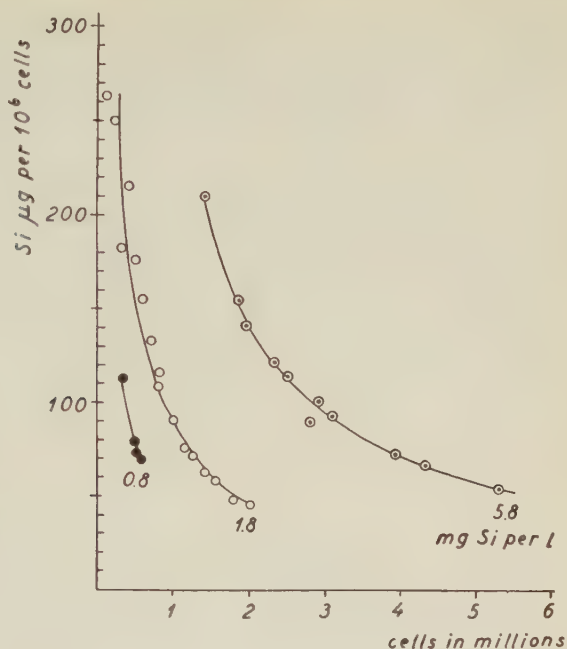
The culture solution was placed in glass bottles with a capacity of 10 l and with their insides covered with paraffin. The bottles were placed at a higher level than the cultures and connected with the culture containers with tubes of polystyrene. When cultivating in a flowing culture solution an ordinary siphon system was started. The current was regulated by means of a Mohr's clip. The rate of the flow was adjusted to approximately 2 l per day. The volume of the culture containers was about 100 ml.

The culture solution was not sterilized in these experiments, but no visible growth of bacteria was observed in the culture containers. This is surely connected with the development of antibiotics by the diatoms.

Four apparatuses with flowing culture solutions were run simultaneously. The cultures were illuminated continuously by means of fluorescent lamps (about 6000 lux).

When the experiment was stopped the diatoms were loosened from the bottom of the container by means of concentrated sulphuric acid. Then the diatom material

Figure 2. The ratio between the silica content expressed as $\mu\text{g Si per } 10^6 \text{ cells}$ and the number of cells of *Bacillaria paradoxa* in experiments with the initial silicon concentration of 0.8, 1.8, and 5.8 mg Si per l.



was divided into two identical portions, one for the counting of cells, the other for the determination of silicon in the cells.

The diatoms in sulphuric acid were evaporated in a platinum crucible and heated till all material was white. Then 1 g of anhydrous Na_2CO_3 was added and fused till melting to give sodium silicate. The sodium silicate was dissolved in 1 l of dist. water. The surplus of sodium carbonate was neutralized by adding hydrochloric acid. The concentration of silicate was determined by the blue method (Bunting 1944).

The other part of sulphuric acid and diatoms was diluted suitably dependent on the number of cells. After a careful shaking 3 ml was placed in a tube chamber with a cover glass as bottom. When the cells were sedimented after about 24 hours they were counted. An inverted microscope was used. The method is the same as that used at quantitative phytoplankton investigations.

Results

Table 2 shows the results for some experiments with *Nitzschia palea* in flowing culture solution. The growth of the diatoms was dependent on the Si-concentration as previously shown (Jørgensen 1952 and 1953). In the last experiment 102.5 million cells were produced in 6 days at 0.5 mg Si per l but 299.0 million cells in 3 days at 5 mg Si per l. The experiments show that the silica content in the cells decreases with increasing number of cells. In

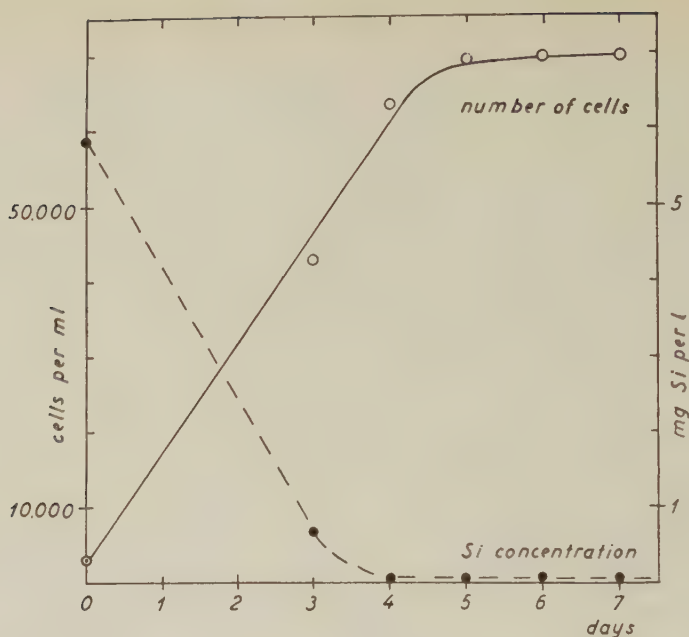


Figure 3. The growth of *Thalassiosira nana* expressed as number of cells per ml in an experiment with the initial silicon concentration of 6 mg Si per l and corresponding values for the silicon concentration in the culture solution during the experiment.

experiments with constant Si-concentration this cannot be due to differences in the growth rate. The only factor that varies with increasing number of cells is that the cells will be closer to each other. *So it is possible that the cells give off a substance that is able to inactivate the assimilation of silica.* When the number of cells increases and the cells are getting closer to each other this inactivation naturally will also increase.

In the series with 0.5 mg Si per l there is an evident decrease in silica content in the cells when the number of cells increases from 1.9 to 9.9 million cells, but very little decrease in the silica content when the number of cells increases from 9.9 to 102.5 million cells. The same is the case in the series

Table 2. *Experiments with Nitzschia palea in flowing culture solution.*

0.5 mg Si per l	Number of cells in millions Si μ g per 10^6 cells	1.9 62	2.3 52	2.9 36	9.9 23	102.5 20
5 mg Si per l	Number of cells in millions Si μ g per 10^6 cells	8.4 26	9.2 38	27.3 18	83.8 16	299.0 14

with 5 mg Si per l when the number of cells increases from 27.3 to 299.0 million cells. This is probably caused by the colonies having reached a point where they cannot be closer to each other and therefore the inactivation will not increase any further. The shape of the curves in figures 1 and 2 shows the same.

When the silica contents of the cells in the two series with different Si-concentration and the same number of cells are compared, it is seen that the values for silica content as regards the greatest number of cells is smallest in the series with 5 mg Si per l. This is because the growth rate is highest at this Si-concentration. Owing to the different growth rate at different Si-concentration it is impossible to show if the silica content in the full-grown cells is dependent on the Si-concentration.

Summary

Investigations of the plankton diatoms in lakes and planktonic and epiphytic diatoms in cultures have shown that the silica content in the cells depends on the number of cells, and the silica content is inversely proportional to the number of cells. This is the case both in ordinary cultures where the Si-concentration and the concentration of other nutrient salts are decreasing when the number of cells increase and in cultures with a flowing culture solution. The decrease in the silica content is probably caused by diatoms giving off a substance to the water which inactivates the silica assimilation.

References

- Bunting, W. E.: Determination of soluble silica in very low concentrations. — *Ing. Eng. Chem. Anal. Ed.* 16: 612. 1944.
- Einsele, W. & J. Grim: Über die Kieselsäuregehalt planktischer Diatomeen und dessen Bedeutung für einige Fragen ihrer Ökologie. — *Zeitschr. f. Botanik*, 32: 545. 1938.
- Jørgensen, E. G.: Effects of different silicon concentrations on the growth of diatoms. — *Physiol. Plant.* 5: 161. 1952.
- Silicate assimilation by diatoms. — *Ibid.* 6: 301. 1953.
- Lund, J. W. G.: Studies on *Asterionella formosa* Hass II. Nutrient depletion and the spring maximum. Part II. — *Journ. of Ecology*, 38: 15. 1950.

Solubility of the Silica in Diatoms

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The wall of the diatom-cell consists of pectin and silica, but it has not yet been definitely established whether the silica is deposited within the substance of the pectin membrane (Mangin 1908), or whether it forms a separate layer of the wall, external to the pectin-layer as Liebig affirms (1928, 1929).

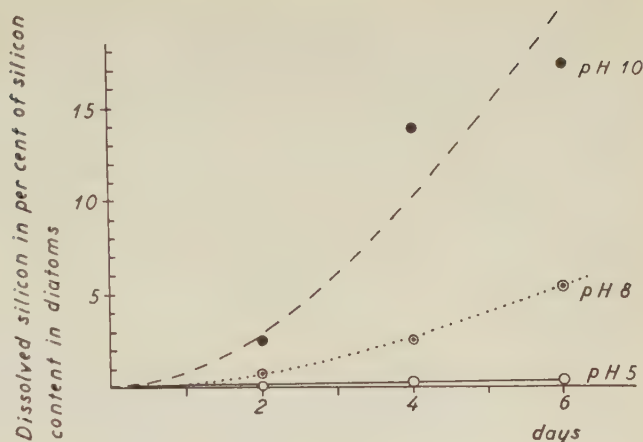
Rogall by chemical analysis and X-ray crystallography showed 1939 that the dead frustules consist of pure silicic acid. Today it is well-known that silicon forms a large number of organic compounds. In other plants several organic silicon compounds are known. Thus Engel (1953) has shown that 80 per cent of the silicon in rye straw is found as a complex between d-galactose and silicic acid. Therefore it would be very remarkable if the silicon in diatoms should occur in a pure inorganic form.

As to the solubility of the silica in diatoms there is a direct conflict in the literature; the problem has been carefully treated by Cooper (1952). Hart (1942) thinks that re-solution of diatomaceous silica is a speedy process in the sea. Atkins (1945) tried to dissolve diatoms in alkaline solutions, from the alkalinity of seawater upwards to pH 11, both at air temperatures and at temperatures about 100° C; but he did not succeed, even with attempts of long duration. Atkins did not during his experiments examine if the diatoms were partly attacked, he only showed that whole cells were not dissolved.

Experiments

In order to elucidate the problem about the solubility of diatom cells a little further some experiments were carried out with two different diatom species. The apparatus was constructed entirely of plastics.

Figure 1. Solubility of the silicon in cells of *Nitzschia linearis* in 6 days at pH 5, 8 and 10 expressed in per cent of the total silicon content.



Experiments with Nitzschia linearis W. Smith. The experiments were carried out at three different pH values, 5, 8 and 10. 10^{-5} -N hydrochloric acid aerated with a 5 per cent carbon dioxide-air mixture was used for experiments at pH 5. No buffer was applied at this pH-value, since adequate quantities of phosphate make the silicon determinations impossible. The alkaline solutions were buffered by means of carbonate-bicarbonate mixtures. That with pH 8 contained 191 mg KHCO_3 and 0.4 mg K_2CO_3 per l and that with pH 10 132 mg KHCO_3 and 46.9 mg K_2CO_3 per l. The solution with pH 8 was aerated with atmospheric air and that with pH 10 was aerated with air free from carbon dioxide. Containers of polystyrene was used in this experiment, each holding 100 ml solution. From a culture of the epiphytic freshwater diatom *Nitzschia linearis*, killed with methyl alcohol, identical quantities of diatoms were added to the containers, and one portion was used for the determination of silica in the cells after the method described in Jørgensen 1955. Each portion contained 5 mg Si. Two, four and six days after the start the Si-concentration in the solutions was determined in 10 ml-samples according to the methods in Jørgensen 1953.

Figure 1 shows the results of the three series. Each point is the average value of duplicate solutions. It is evident that the cells of this diatom are soluble in water and that the solubility is dependent on the pH of the solution. Correns (1940) correspondingly shows that the solubility of silica in water depends on the pH with minimum values at pH 3 and increasing to pH 11, which is the highest pH value he has examined. In the present experiment during six days 0.3 per cent of the silicon in *Nitzschia linearis* was dissolved at pH 5, 5.3 per cent at pH 8, and 17.2 per cent at pH 10.

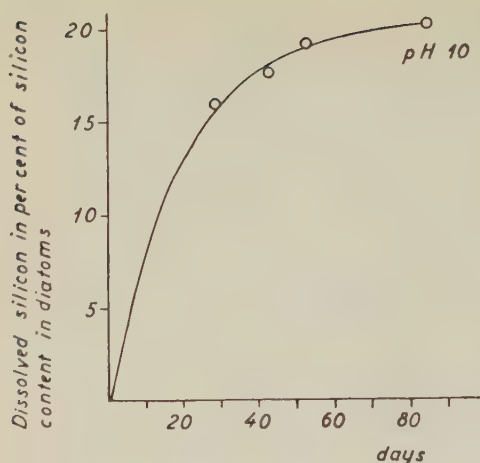


Figure 2. Solubility of *Nitzschia linearis* in 85 days at pH 10.

Figure 2 shows a similar experiment at pH 10 carried out in polyethylene bottles containing 500 ml solution. To each bottle a portion of cells of *Nitzschia linearis* was added containing 2.1 mg Si. In this experiment the diatoms were filtered from the solution and added to new solutions after each silicon determination. This was done to avoid that the silicon concentration in the solution should influence the dissolution of silicon in the diatoms. The experiment lasted 85 days. The points are the average values of duplicate solutions. In this experiment only 5.2 per cent of the silicon content was dissolved in six days while 17.2 per cent was dissolved in the first experiment in the same period. Thus the silica compounds of cells from the same species are sometimes easier to hydrolyze than at other times. In 85 days 20 per cent of the silicon in the cells was dissolved. The shape of the curve shows that the rate of solution is much higher at the beginning of the experiment than at the end. This shows that the silica compounds of the cells are hydrolyzed at different rates. Some of the compounds are easy to hydrolyze, others are hydrolyzed with great difficulty.

Experiments with Thalassiosira nana Lohmann. Similar experiments were carried out with a marine planktonic species, *Thalassiosira nana*. The experiments were carried out in polyethylene bottles containing 500 ml. Portions of cells containing 960 μg Si were added to each bottle in both experiments. Also in this experiment duplicate solutions were used. Figure 3 in the same way as figure 1 for *Nitzschia linearis* shows that the solubility depends on pH in the solution. In 8 days 1.3 per cent of the silicon in the cells was dissolved at pH 4, 4.2 per cent at pH 8, and 16.4 per cent at pH 10. In 14 days 5.4 per cent at pH 8 and 32.3 per cent at pH 10 were dissolved.

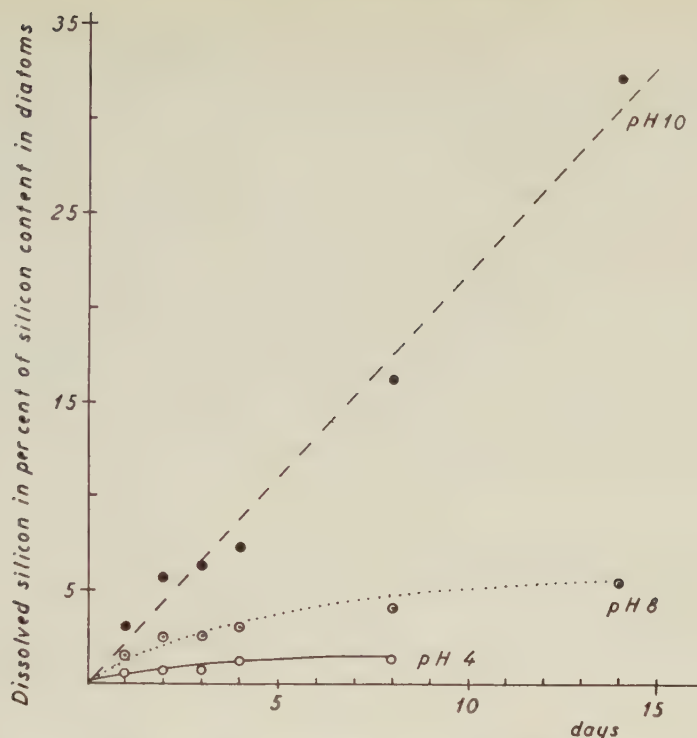


Figure 3. Solubility of the silicon in cells of *Thalassiosira nana* in 14 days at pH 4, 8 and 10 expressed in per cent of the total silicon content.

In figure 4 an experiment of longer duration with *Thalassiosira nana* and four replicate solutions at pH 10 is pictured. In 14 days 32.3 per cent of the silicon in the cells was dissolved, the same quantity as in the first experiment. In this experiment there is a simple proportionality between the solubility and the time. After 37 days when the experiment was stopped 97 per cent of the silicon was dissolved. So this diatom will be completely dissolved at pH 10.

The experiments with the two species show that the silicon compounds in the cells are soluble in water and that the rate of solubility depends on the pH, being greatest at the highest pH value. So the silicon compounds are found in forms in the cells that are more or less easy to hydrolyze. In *Thalassiosira* all the silicon compounds are easy to hydrolyze. In *Nitzschia linearis* some parts are easy, others difficult to hydrolyze.

Other factors affecting the solubility of diatom cells. According to solubility experiments with diatoms by King & Davidson (1933) diatoms heated in water before the experiment were not dissolved so fast as diatoms not

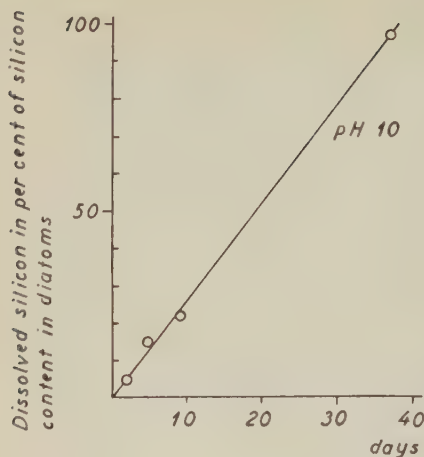


Figure 4. Solubility of *Thalassiosira nana* in 37 days at pH 10.

heated in this way. The authors supposed that an enzyme in the diatoms affecting the solution of silicon was inactivated in hot water. Several experiments carried out in connexion with the present investigations and in the same way as King & Davidson could not confirm their observations.

Summary

Experiments carried out with the diatoms *Nitzschia linearis* and *Thalassiosira nana* show that the silicon in the cell walls is soluble in water and that the rate of solubility depends on the pH of the solution. The rate increases with increasing pH. At pH 8 not much more than 5 per cent of the silicon in the cells was dissolved in both species during the experiments, at pH 10 *Thalassiosira* was dissolved completely. In the case of *Nitzschia linearis* 20 per cent of the silicon was dissolved at pH 10 during the experiment. Thus the silicon compounds are found in the diatom cells in forms which are hydrolyzed at different rates. The silicon compounds in *Thalassiosira* are easy to hydrolyze at pH 10. In *Nitzschia linearis* some parts are easy to hydrolyze, others very difficult.

References

- Atkins, W. R. G.: Autotrophic flagellates as the major constituent of the oceanic phytoplankton. — *Nature* 156: 446. 1945.
- Cooper, L. H. N.: Factors affecting the distribution of silicate in the North Atlantic ocean and the formation of North Atlantic deep water. — *Journ. Mar. Biol. Ass. United Kingdom* 30: 511. 1952.

- Correns, C. W.: Über die Löslichkeit von Kieselsäure in schwach sauren und alkalischen Lösungen. — *Chemie der Erde* 13: 92. 1940.
- Engel, W.: Untersuchungen über die Kieselsäureverbindungen im Roggenhalm. — *Planta* 41: 358. 1953.
- Hart, T. J.: Phytoplankton periodicity in Antarctic surface waters. — *Discovery Reports* 21: 261. 1942.
- Jørgensen, E. G.: Silicate assimilation by diatoms. — *Physiol. Plant.* 6: 301. 1953.
— Variations in the silica content of diatoms. — *Ibid* 8: 000. 1955.
- King, E. J. & Davidson, V.: The biochemistry of silicic acid. IV. Relation of silica to the growth of phytoplankton. — *Biochem. Journ.* 27: 1015. 1933.
- Liebisch, W.: *Amphitetras antediluviana* Ehrbg., sowie einige Beiträge zum Bau und zur Entwicklung der Diatomeenzelle. — *Zeitschr. f. Bot.* 20: 225. 1928.
— Experimentelle und kritische Untersuchungen über die Pektinmembran der Diatomeen unter besonderer Berücksichtigung der Auxosporenbildung und der Kratikularzustände. — *Ibid.* 22: 1. 1929.
- Mangin, M. L.: Observations sur les diatomées. — *Ann. Sci. nat. Bot.* IX, 8: 177. 1908.
- Rogall, E.: Über die Feinbau der Kieselmembran der Diatomeen. — *Planta* 29: 279. 1939.

Two Fatty Acid Requiring Mutants of *Ophiostoma multiannulatum*

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In fungi a few cases have been reported where a fatty acid constitutes an essential ingredient of the nutrient medium. Thus it has been shown by Benham (1941, 1947) that *Pityrosporum ovale* does not grow at all without oleic acid, and by Yusef (1953) that three species of *Polyporus* are greatly stimulated by this acid. Lein and co-workers have studied induced mutations of *Neurospora crassa* which exhibit an absolute requirement for certain fatty acids (Lein and Lein 1949, 1950, Lein, Puglisi and Lein 1953).

Some years ago a number of fatty acid requiring mutants were isolated from the ascomycete *Ophiostoma multiannulatum* in this laboratory. Their physiological characters were not studied at that time, however, and only two strains were retained as stock cultures. These strains have now been more closely investigated. The results show that they differ in some essential points from the *Neurospora* mutants referred to above.

Material and Methods

The strains in question, Nos. 2374 and 2377, were produced in March 1950 by ultraviolet irradiation of wild type conidia. They could be kept alive by repeated transfers on «complete agar medium» without adding any fatty acids, although under such conditions the mycelium remained extremely thin and scarcely visible

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on the agar surface, mainly growing submerged and not forming any aerial hyphae. No conidia were produced.

Back-mutations to wild type occurred now and then in both strains and appeared as spots of a much denser mycelium, easily distinguished by its entirely different mode of growth.

Since both mutants, in contrast to most *Ophiostoma* strains, were unable to grow in conidial form, their growth could not be photometrically estimated. The dry weight of mycelium produced in a liquid nutrient solution was therefore taken as a measure of growth. Small pieces of a plate culture on complete agar (Fries 1948) were used as inocula and transferred to 100 ml. Jena flasks containing the nutrient solution to be studied, 25 ml. per flask. «Modified medium 3» (Fries 1948) was used as the basal medium, although with ammonium sulphate (3.6 g/l.) instead of ammonium tartrate. The flasks were incubated at + 25 °C for a suitable period of time. The developed mycelia were removed, washed with hot water, dried at + 100° C, and weighed.

Experiments

Good growth was obtained by adding appropriate fatty acids to the synthetic basal medium (Tables 1 and 2). All short-chain acids tested, possessing 10 carbon atoms or less, *viz.* acetic, valeric, caproic, isocaproic, heptanoic, capric, succinic, fumaric, azelaic, sebacic, malic, tartaric, and citric acid, were completely incapable of supporting growth. No free acids with 11 to 15 carbon atoms in the molecule were available for testing. All acids with 16 or 18 carbon atoms, however, the effects of which could be investigated, supported growth of the two mutant strains. In the concentrations tested the saturated acids, *viz.* palmitic and stearic acid, promoted growth more efficiently than the unsaturated ones in strain No. 2377, whereas the unsaturated oleic acid was most active in strain No. 2374 (Table 2). Linoic and linolenic acid proved less satisfactory.

Table 1. *Growth of the two mutant strains with various supplements added to the basal solution.* — The basal medium contained ammonium tartrate (5 g/l) instead of ammonium sulphate. Initial pH varied from 5.25 to 5.35. The incubation time was 30 days for mutant No. 2374 and 29 days for mutant No. 2377. Average values from three cultures.

Supplements per flask	Mycelium per flask, dry weight in mg	
	No. 2374	No. 2377
None	0	0
Malt extract (Vitrum) 0.2 % + Bacto yeast extract 0.02 %	0	0
Hydrolyzed casein (NZ-case) 0.2 % + 1 mg 1-tryptophane	0	0
Hydrolyzed casein (NZ-case) 0.2 % + 1 mg 1-tryptophane + 0.15 ml. Tween 80 (oleic acid derivative)	37.5	47.0

Table 2. *Growth of the two mutant strains with various fatty acids.* — All acids were added in form of sodium salts. Initial pH 5.6—5.9. Time of incubation 20 days. Average values from five cultures.

Fatty acids added (mg per flask)	Mycelium per flask, (dry weight in mg.)		Final pH	
	No. 2374	No. 2377	No. 2374	No. 2377
None	0	0	5.4	5.2
Palmitic, 12.5	5.2	16.3	3.7	2.9
Stearic, 12.5	4.8	15.7	3.7	2.7
Oleic, 12.5	9.1	11.3	3.3	2.8
Linic + linolenic, 4.3	1.8	1.7	3.4	3.2

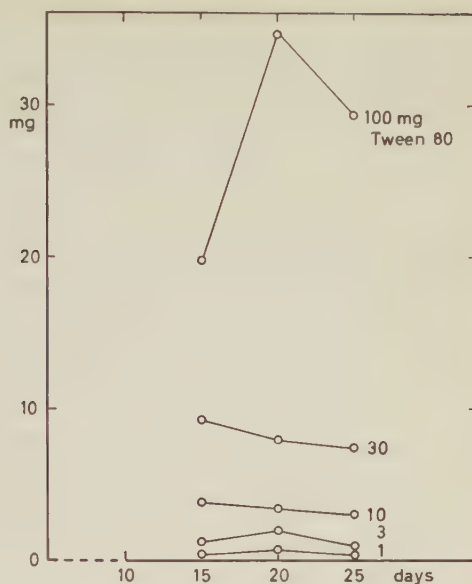
The best way of supplying the cultures with these long-chain fatty acids was in the form of »Tweens», i.e. as polyoxyethylene-sorbitan monoderivatives, which are much more soluble in water than the free acids (Table 3). In this form oleic acid proved to be the generally most active one of those tested. No. 2374 responded stronger to the saturated acids as sorbitan derivatives than did No. 2377. The former, but probably not the latter, was able to utilize lauric acid (13 carbon atoms) as Tween 20, although this acid appeared to possess a certain toxicity noticeable in higher concentrations (Tables 3 and 4).

With Tween 80 as the source of oleic acid an investigation was made as to how much mycelium could be produced by the aid of a given quantity of Tween (Figure 1). Since Tween 80 contains c. 25 per cent of oleic acid (Davis 1947) it is evident that the dry weight of mycelium produced is somewhat higher than the quantity of oleic acid supplied. It may thus be concluded

Table 3. *Growth of the two mutant strains with various Tweens.* — The basal medium contained no glucose. Ten drops (=0.15—0.20 ml.) of Tween 40 weighed c. 18 mg, ten drops (=0.15 ml.) of Tween 80 c. 16 mg. Initial pH 5.4—5.7. Incubation time 31 days. Average values from five cultures.

Tween added in drops per flask	Mycelium per flask, dry weight in mg	
	No. 2374	No. 2377
None	0	0
Tween 20 (Laurate): 5 drops	4.3	1.0
10 »	1.5	0.4
Tween 40 (Palmitate): 5 »	2.7	3.1
10 »	9.8	3.3
Tween 60 (Stearate): 5 »	2.5	3.3
10 »	12.0	5.1
Tween 80 (Oleate): 5 »	8.2	7.4
10 »	10.2	11.1

Figure 1. *The production of mycelium by mutant No. 2377 with graded doses of Tween 80.* The figures to the right indicate the quantity of Tween 80 in mg added to each flask. Each point represents the average of five cultures.



that oleic acid does not play the role of a vitamin-like growth-factor, nor does it serve as the sole source of carbon and/or energy. Therefore, the significance of the glucose present in the medium was investigated.

In the following experiments strain No. 2377 was used as the chief test object, since it provided a more uniform material for inoculation than No. 2374. From the experiments summarized in Tables 3 and 5 it can be seen that growth is possible with Tween 80 as the only carbon source, although it is considerably increased by glucose. Succinate improved the effect of Tween 80 as well as that of Tween 80 plus glucose. This might be due to the buffering capacity of succinate, but other interpretations are also conceivable. A similar beneficial effect was produced by tartrate, fumarate, malate, citrate, aspartate, and glutamate.

Table 4. *Growth of mutant No. 2377 with Tweens and glucose in various combinations.* — The basal medium contained no glucose. Initial pH 5.70–5.85. Incubation time 13 days. Average values from four cultures.

Supplements per flask	Mycelium per flask, dry weight in mg with						
	Tween 20	Tween 40		Tween 60		Tween 80	
	5 drops	10 drops	20 drops	10 drops	20 drops	10 drops	20 drops
None	0.4	1.1	0.7	1.0	0.8	1.3	0.3
Glucose, 500 mg	0.4	4.9	5.7	6.4	8.2	14.3	13.8

Table 5. *Growth of mutant No. 2377 with Tween 80, succinate and glucose in various combinations.* — Basal medium without glucose. Time of incubation 20 days. Average values from eight cultures.

Additions per flask			Mycelium per flask, dry wt. in mg
Tween 80, 25 mg	Sodium succinate, 229 mg	Glucose, 500 mg	
—	—	—	0.1
+	—	—	1.3
—	+	—	0.1
+	+	—	3.1
—	—	+	0.1
+	—	+	11.8
—	+	+	0.2
+	+	+	22.7

A more extensive experiment with various combinations of carbon sources confirmed the above results (Table 6). In judging the figures obtained, however, it is necessary to keep in mind that sodium palmitate was not completely dissolved in the nutrient solution unless Tween 80 was simultaneously present. This explains, at least partly, the high yield obtained with both these substances combined. The buffering effect of sodium succinate is particularly clear in the glucose series. A glucose determination showed that, in the series with Tween 80 and palmitate, 25 per cent of the glucose, i.e. 62.5 mg per flask, had been consumed after 25 days.

Table 6. *Growth of mutant No. 2377 with Tween 80, palmitate, succinate, and glucose in various combinations.* — Basal medium without glucose. Average values from five cultures.

Additions per flask				Mycelium per flask, dry wt. in mg			pH	
Tween 80, 25 mg	Sodium palmitate, 13.6 mg	Sodium succinate, 28.6 mg	Glucose, 500 mg	15 days	20 days	25 days	Initial	Final
—	—	—	—	0	0.1	0.1	4.3	4.3
+	—	—	—	1.8	1.9	1.3	4.3	3.8
—	+	—	—	0.3	1.7	1.2	5.7	5.6
—	—	+	—	0	0	0	5.7	5.9
+	+	—	—	4.9	7.2	6.0	5.8	5.3
+	—	+	—	3.4	3.4	2.6	5.7	7.0
—	+	+	—	0.2	1.9	1.1	5.7	6.8
+	+	+	—	3.8	4.6	3.7	5.9	6.9
—	—	—	+	0	0	0	4.2	4.2
+	—	—	+	8.4	10.8	11.2	4.2	2.9
—	+	—	+	4.6	5.9	6.3	5.6	3.3
—	—	+	+	0.5	0	0	5.7	5.5
+	+	—	+	23.9	26.4	29.6	5.7	2.7
+	—	+	+	13.1	23.8	25.0	5.7	3.0
—	+	+	+	1.8	2.6	4.1	5.7	4.7
+	+	+	+	30.1	37.5	56.7	5.8	2.8

In the presence of larger amounts of Tween 80 and sodium palmitate the mycelial production was approximately proportional to the quantity of glucose added, the economic coefficient being about the same as that obtained with Tween 80.

Discussion

From the experiments now described it seems quite evident that mutant strain No. 2377 (and probably also No. 2374) represents a physiological type different from all other fatty acid requiring mutants earlier investigated. Unlike the *Neurospora* mutant No. S-210 (Lein and Lein 1950) it is incapable of growing with acetate, and unlike the *Neurospora* mutant No. S-11 (Lein and Lein 1949, Lein, Puglisi and Lein 1953) it grows with both saturated and unsaturated long-chain acids.

Although the fatty acids are required in such amounts that they must be utilized not only as building material but also as sources of energy, growth without sugar is very poor. Since the wild type is able to get on with sugar alone, something seems to have been blocked or damaged in the biochemical equipment of these mutants, which can be compensated for by the supply of certain fatty acids. It appears useless, however, to speculate on the biochemical mechanism behind these unusual nutritional requirements, until more is known about how the sugar and the fatty acids are metabolized by the mutants.

It is interesting to note that the lipophilic fungus *Pityrosporum ovale* (Benham 1947) seems to represent a wild type parallel to No. 2377, in so far as it displays the same need for a fatty acid plus glucose in order to produce a satisfactory growth.

Summary

The mutants Nos. 2374 and 2377 of *Ophiostoma multiannulatum* could be cultivated in a glucose-mineral-salt solution if palmitic, stearic or oleic acid were included in the medium. Lauric, linoic, and linolenic acid proved less satisfactory, and no acid with a carbon atom number below 10 showed any activity.

The growth was poor unless glucose (or some other suitable carbohydrate) was present together with the fatty acid. Each compound was required in such quantity as to suggest a utilization not only as a nutrient but also as a source of energy.

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References

- Benham, R.: Cultural characteristics of *Pityrosporum ovale* — a lipophilic fungus. Nutrient and growth requirements. — *Proc. Soc. Exp. Biol. and Med.* 46: 176. 1941.
- Biology of *Pityrosporum ovale*. — *Biology of pathogenic fungi*, ed. by W. J. Nickerson. Waltham, Mass. 1947.
- Davis, B. D.: The preparation and stability of fatty acid-free polyoxyethylene sorbitan monooleate («Tween» 80). — *Arch. Biochem.* 15: 359. 1947.
- Fries, N.: Viability and resistance of spontaneous mutations in *Ophiostoma* representing different degrees of heterotrophy. — *Physiol. Plant.* 1: 330. 1948.
- Lein, J. & Lein, P. S.: Studies on a *Neurospora* mutant requiring unsaturated fatty acids for growth. — *Journ. Bact.* 58: 595. 1949.
- & — The production of acetate from fatty acids by *Neurospora*. — *Journ. Bact.* 60: 185. 1950.
- Lein, J., Puglisi, T. A. & Lein, P. S.: Unsaturated fatty acid metabolism in *Neurospora*. — *Arch. Biochem. and Biophys.* 45: 434. 1953.
- Yusef, H. M.: The requirements of some *Hymenomycetes* for essential metabolites. — *Bull. Torrey Bot. Club* 80: 43. 1953.

The Significance of Thiamin and Pyridoxin for the Growth of the Decotylished Pea Seedling

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Introduction

In the experiments earlier described (Fries 1954), which demonstrated the significance of arginine, glycine, and adenine as limiting factors in the growth of etiolated, decotylished pea seedlings, a number of vitamins were always present in the basal medium. Of these vitamins thiamin (B₁) and niacin were known to be indispensable for pea root cultures (Addicott and Bonner 1938), whereas the others had been reported as partially limiting factors for decotylished pea seedlings (Kögl and Haagen-Smit 1936, Saubert-v. Hausen 1948).

A few experiments were then performed in order to throw some light upon the rôle of the vitamins in the medium. It was found that the etiolated and decotylished seedlings did not grow without vitamins being added, but that the only vitamins required during an experiment were thiamin and pyridoxin (Fries 1955). The experiments reported in the present paper confirm these results and demonstrate certain qualitative and quantitative differences between the two vitamins as regards their activity.

Material and Methods

All seedlings were obtained from »Torsdag III» pea seeds of the 1953 harvest. The sterilisation and soaking of the seeds as well as the arrangement of the experiments in general was carried out in accordance with earlier, detailed descriptions

(Fries 1953, 1954). When not otherwise indicated the seedlings were cultivated for 15 days in ordinary culture tubes with 10 ml. nutrient agar medium. When Ryan tubes were employed, they contained 15 ml. of the same medium.

The composition of the basal nutrient solution, solidified with 1.5 per cent agar, was the same as earlier described, except that the two vitamins thiamin and pyridoxin (and in the two experiments of Table 1, niacin) were omitted. Since these vitamins must represent the only limiting factors of the medium, the three partially limiting metabolites, *arginine, glycine and adenine (each 0.3 mM) were always included in the solution.*

The cultures were kept in darkness, if nothing to the contrary is stated. When they were exposed to the light, this means that they were irradiated with white light for 14 hours a day. The light source was a combination of an incandescent lamp and a mercury lamp (Åberg 1943, p. 27), which produced c. 4,000 lux at the distance where the culture were placed. However, the cotton plugs shaded the seedlings from a great part of the direct irradiation. The lower part of the tubes were covered with black paper to prevent light from reaching the roots.

Experiments

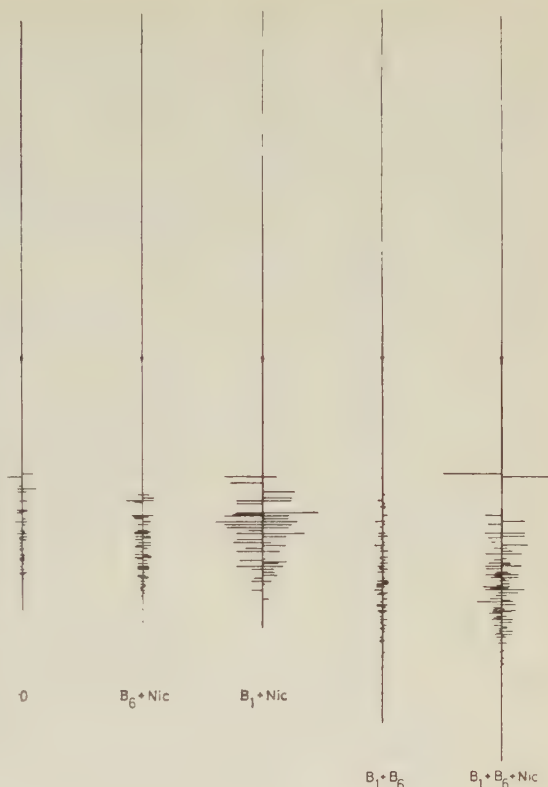
A. Introductory experiments

The result of an experiment with the three vitamins, thiamin, pyridoxin and niacin, in various combinations has already been briefly reported and commented on in an earlier paper (Fries 1955). A few more words may be said about the data obtained from this experiment (No. 1200 in Table 1). As the figures show, thiamin and pyridoxin, but not niacin, are necessary for the growth of the main root of the decotylised seedling. However, if the growth of lateral roots is considered, niacin (in addition to thiamin) seems to be more important than pyridoxin (Figure 1).

The excised roots apparently developed equally well with as without vitamins during the 15 days of the experiment. The difference in average length of laterals is not statistically significant. With the three vitamins added the attached roots grew at the same rate as the excised ones, the most striking difference being not the number or average length of laterals but the distribution of the laterals along the main root axis.

The importance of thiamin and pyridoxin also appeared from experiments with Ryan tube cultures (Figure 2). The cultures of decotylised seedlings need no comment; the root cultures, just as in the above reported experiment No. 1200 (Table 1) did not show any response to the two vitamins during the 15 days of the experiment (Expt. 1189, Figure 2). However, if excised roots are grown in Ryan tube culture during a longer period a difference in growth rate (not shown in Figure 2) between those with and

Figure 1. *Schematic reproduction of decotylised seedlings cultivated in test tubes for 15 days with additions of various vitamins. Each plant in the figure is a composite picture of seven plants picked at random from the series, the shoot and the main root length being the average of these seven shoots and main roots. The insertion point of each lateral root occupies the same relative position on the average main root as on the real main root to which it belongs. Otherwise all sizes are $\frac{2}{5}$ of the natural size.*



those without the two vitamins becomes visible after 15 to 20 days. Since the excised roots do not require pyridoxin this difference is probably exclusively based on the effect of thiamin.

Light makes it possible for the seedling to synthesize all necessary vitamins. However, as demonstrated convincingly by Saubert-v. Hausen (1948), the synthesizing power of the decotylised seedling even in full daylight is far from optimum during a long period following germination. Without sugar in the medium no growth occurs even with vitamins added. With 2 per cent sucrose, however, growth is possible in light without any vitamins, although it only slowly surpasses the growth in darkness with vitamins (Expt. 1208 in Table 1). In the former case the shoot is favoured, in the latter the root system. A peculiar fact is that the number (but not the average length) of the lateral roots is greater in darkness. Whether the roots are directly affected or whether the effect is produced indirectly through mediation of the shoot is difficult to determine, since it was impossible to screen off the submerged part of the seedlings completely from light.

Table 1. *Effect of thiamin (B₁), pyridoxin (B₆), and niacin amide (Nc) on the growth of decotylised pea seedlings and excised pea roots.*

Unless otherwise stated in the »Particulars» column the experimental material consisted of decotylised plants cultivated in the dark. »Roots» means excised roots with the hypocotyl attached. The concentration of each added vitamin 100 µg./l. Niacin was omitted from the basal medium. Shoot length, root length (incl. hypocotyl), and dry weight of the seedlings at the start of the experiments were in Expt. 1200: c. 6 mm, 31 mm, and 4.8 mg., in Expt. 1208: c. 5 mm, 27 mm, and 5.9 mg., respectively. Each series in Expt. 1200 comprised ten, in Expt. 1208 eight cultures.

Expt. No.	Vitamins added	Particulars	Time in days	Length in mm			No. of lateral roots	Dry weight in mg.		
				Shoot	Main root	Lateral roots		Shoot	Root system	Whole plant
1200	None ...	—	15	111 ± 3	84.7 ± 4.2	10.9	7.1	7.1	4.5	11.6
	B ₆ Nc ...	—	15	114 ± 3	90.8 ± 3.6	21.6	8.6	7.3	4.8	12.1
	B ₁ Nc ...	—	15	116 ± 3	91.4 ± 6.9	54.3	6.9	6.7	5.0	11.7
	B ₁ B ₆	—	15	116 ± 1	123.0 ± 2.6	15.4	10.6	7.3	5.1	12.4
	B ₁ B ₆ Nc	—	15	113 ± 2	136.5 ± 2.5	44.0	11.2	7.3	5.6	12.9
	None ...	Roots	15	—	134.0 ± 3.9	88.3	17.6	—	14.1	—
	B ₁ B ₆ Nc	Roots	15	—	142.4 ± 1.0	104.0	17.1	—	14.3	—
1208	None ...	—	10	65 ± 3	79.4 ± 5.6	8.6	4.7	5.3	4.4	9.7
		—	15	98 ± 6	86.5 ± 4.5	15.4	8.9	6.1	4.7	10.8
		Light	10	68 ± 7	87.3 ± 3.6	15.8	4.3	5.2	5.3	10.5
		Light	15	111 ± 9	95.0 ± 2.9	23.3	7.4	7.7	5.1	12.8
	B ₁ B ₆ Nc	—	10	65 ± 4	108.4 ± 3.7	9.9	4.7	5.0	4.9	9.9
		—	15	102 ± 5	119.9 ± 5.7	63.9	11.5	6.4	5.1	11.5
		Light	10	63 ± 5	111.9 ± 3.7	12.7	2.7	4.9	5.9	10.8
		Light	15	126 ± 7	135.3 ± 5.8	83.8	8.7	8.2	5.9	14.1
	B ₁ Nc ...	—	15	97 ± 7	97.8 ± 2.8	73.6	6.1	6.1	5.0	11.1

Table 2. *Effect of thiamin (B₁) on the growth of decotylised pea seedlings.*

The medium contained pyridoxin in both experiments. The concentration of each added vitamin 100 µg./l. Shoot length, root length (incl. hypocotyl), and dry weight of the seedlings at the start of the experiments were in Expt. 1201: c. 6 mm, 30 mm, and 7.0 mg., in Expt. 1209: c. 3 mm, 26 mm, and 6.1 mg., respectively. Each series comprised ten cultures. For details of the injection technique see the text.

Expt. No.	Vitamins added	Time in days	Length in mm			No. of lateral roots	Dry weight in mg.		
			Shoot	Main root	Lateral roots		Shoot	Root system	Whole plant
1201	None B ₁ in the medium	15	127 ± 2	103.8 ± 2.7	42.3	13.5	7.9	5.2	13.1
		20	132 ± 3	109.2 ± 2.0	46.9	13.8	9.3	5.6	14.9
		15	128 ± 3	144.5 ± 3.2	88.8	15.5	8.2	5.9	14.1
		20	146 ± 4	154.2 ± 3.5	133.5	17.0	10.1	6.2	16.3
1209	None B ₁ in the medium None (H ₂ O in capillary) B ₁ in capillary ...	15	89 ± 5	76.8 ± 3.7	12.0	6.2	5.6	4.4	10.0
		15	109 ± 3	128.4 ± 3.5	42.8	10.7	6.6	5.0	11.6
		15	92 ± 4	76.0 ± 3.0	12.3	6.0	5.6	4.2	9.8
		15	102 ± 3	88.1 ± 2.3	16.0	7.1	6.1	4.8	10.9

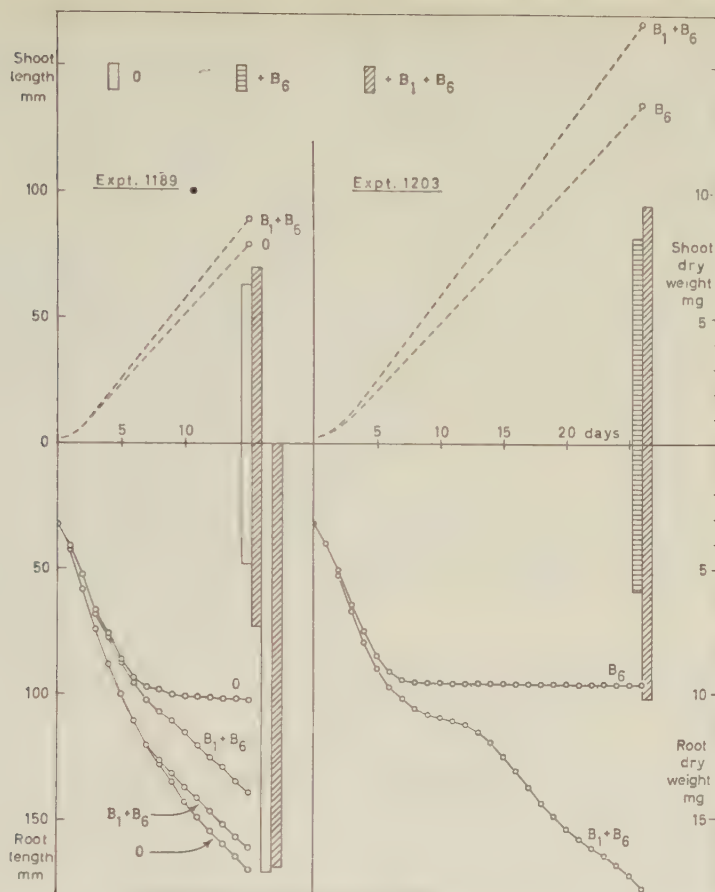


Figure 2. *Effect of thiamin (B_1) and pyridoxin (B_6) on the growth of decotylised seedlings and excised roots cultivated in Ryan tubes. Only the final length of the shoot was recorded. Both vitamins were tested in concentrations of 100 $\mu\text{g./l.}$ ($=1.5 \mu\text{g. per culture}$). The number of cultures per series was in Expt. 1189: 12 in both series without vitamins, 8 in the series with seedlings and vitamins, and 11 in the series with excised roots and vitamins, and in Expt. 1203: 9 in the series with B_6 and 6 in the series with B_1+B_6 .*

B. The effect of thiamin

The rôle of the time factor in the effect of thiamin is shown in Table 2 (Expt. 1201) and Figure 2. It appears from the table that in ordinary culture tubes the development of the decotylised seedlings in the absence of thiamin is almost completely stopped already after 15 days. Five days later no significant increase could be noted either in length of shoot, main root or laterals, or in the number of laterals. With thiamin the seedlings continued

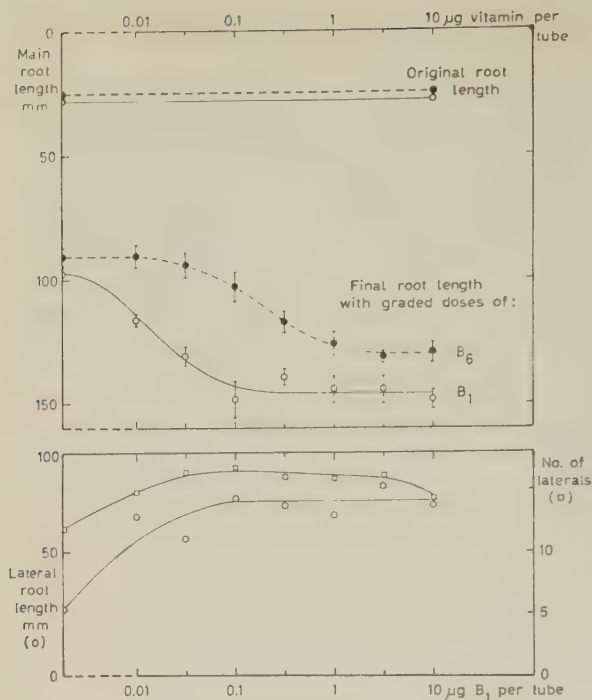


Figure 3. *Effect of thiamin (B₁) on the root system of decotylised pea seedlings.* The upper diagram shows the length of the main root attained after 15 days with different doses of B₁. The broken line shows a corresponding curve for B₆. In the lower diagram the average length of the laterals per root system (circles) is given, as well as the average number of laterals per root system (squares). Most points represent average values from six cultures.

to grow in all these respects, although the elongation of the main root was probably impeded by the culture conditions.

The growth curve of the attached main root in a Ryan tube culture has the same shape as those obtained with arginine, glycine or adenine as the limiting factor (Fries 1955, pp. 25, 33 and 34), except that the growth stops more definitely in the absence of thiamin (Figure 2).

In all the experiments with thiamin the same concentration of 100 µg./l. was tested. This is obviously several times more than actually necessary, since the same effect is produced by a ten times lower concentration, at least in an experiment of 15 days' duration (Figure 3). The critical thiamin level seemed to be much higher for the main root than for the lateral roots, which developed quite normally with only 1 µg./l., i.e. 0.01 µg. per tube, of thiamin. No inhibitory or otherwise deleterious effects could be observed in cultures with very high doses of thiamin.

Even in the presence of other vitamins and with sucrose as a supplementary source of energy light cannot completely compensate for a lack of thiamin. It is true that the shoot develops better in all respects without thiamin in light than with this vitamin in darkness, but the root system does not seem to benefit from the irradiation even during a comparatively long

Table 3. *The significance of light for the response of the seedlings to thiamin (B_1).*

Concentration of thiamin, when added, and of pyridoxin 100 $\mu\text{g./l.}$ Each series comprised eight cultures.

Additions	Condi- tions	Incubation time in days	Length in mm			No. of lateral roots	Dry weight in mg.		
			Shoot	Main root	Lateral roots		Shoot	Root system	Whole plant
None ...	Dark	15	80 \pm 7	71.0 \pm 5.0	3.5	2.8	5.2	4.8	10.0
	Dark	30	120 \pm 9	76.7 \pm 5.4	3.0	1.7	7.1	5.4	12.5
	Light	15	74 \pm 9	74.6 \pm 4.0	16.4	2.0	5.7	5.9	11.6
	Light	30	149 \pm 8	68.8 \pm 8.5	22.2	3.2	9.2	5.3	14.5
B_1	Dark	15	73 \pm 7	72.7 \pm 9.5	8.3	3.6	4.7	4.6	9.3
	Dark	30	133 \pm 11	107.6 \pm 8.7	72.4	8.1	7.7	6.7	14.4
	Light	15	67 \pm 6	110.4 \pm 3.4	13.3	2.0	4.9	6.1	11.0
	Light	30	194 \pm 13	121.3 \pm 7.8	102.3	5.3	12.2	7.5	19.7

time of incubation (Table 3). As a matter of fact, the main root length remained unchanged and the dry weight of the root system decreased in irradiated seedlings without thiamin during the second 15 days' period. The best general development was attained in the series with both light and thiamin.

If we assume that a synthesis of thiamin is induced in the shoot through irradiation, it is surprising that none of this thiamin reaches the root system. As was shown in Figure 3, extremely small quantities are sufficient for producing a clear response in this part of the seedling. In order to find out if a transport of thiamin could occur from shoot to root at this stage of development in seedlings cultivated under these conditions, the following experiment was performed.

Glass capillaries closed at one end and having an inner diameter of 0.1—0.2 mm. and a length of 10—12 cm. were filled with a concentrated sterile solution of thiamin, 10 mg./ml. The open end of the capillary was introduced into the epicotyl of an etiolated, decotylised pea seedling six days old and growing in an ordinary tube culture. This operation was performed under a microscope with the culture tube in horizontal position. The capillary was squeezed in through a hole in the epidermis just above the second node, this hole having been made immediately before by means of a sharp needle. The upper, closed end of the capillary which protruded a few centimeters from the culture tube was then broken, so as to permit the thiamin solution to be absorbed by the epicotyl tissue. Each capillary contained c. 0.0022 ml. solution, i.e. 2.2 $\mu\text{g.}$ thiamin.

Another series of seedlings was provided with capillaries containing distilled water, which was injected in the same way as described above. These

Table 4. *Effect of graded amounts of pyridoxin on the growth of decotylised pea seedlings.*

The medium contained 100 µg./l. of thiamin, i.e. 1 µg. per culture tube. The seedlings had a shoot length of c. 4 mm, a root length of 24 mm, and a dry weight of 5.7 mg. at the start of the experiment. Each series comprised eight cultures. Incubation time 15 days.

µg. pyridoxin per culture tube	Length in mm			No. of lateral roots	Dry weight in mg.		
	Shoot	Main root	Lateral roots		Shoot	Root system	Whole plant
0	104 ± 3	90.3 ± 3.6	51.4	7.1	6.2	4.0	10.2
0.01	100 ± 5	90.3 ± 5.0	43.8	6.5	6.4	4.3	10.7
0.03	102 ± 5	92.5 ± 4.7	51.6	7.6	6.5	4.2	10.7
0.1	108 ± 1	103.0 ± 6.5	52.6	7.5	6.7	4.6	11.3
0.3	106 ± 3	117.3 ± 3.8	42.1	9.9	6.4	4.6	11.0
1	108 ± 2	126.1 ± 4.9	51.9	11.8	6.8	5.0	11.8
3	108 ± 4	131.4 ± 2.2	56.9	11.9	6.9	4.8	11.7
10	107 ± 5	129.1 ± 3.6	49.6	11.1	6.8	5.0	11.8

cultures served the purpose of making sure that the mechanical irritation caused by the capillary did not produce any special effects on the seedlings.

Since the capillaries were empty at the end of the experiment the quantity of thiamin supplied, c. 2.2 µg. must have been absorbed by the shoot. As appears from Table 2 (Expt. 1209), however, this quantity of thiamin caused a considerably weaker effect in the seedling than the smaller amount of thiamin present in the medium of another series. Thus it appears that under the prevailing circumstances thiamin is distributed and utilized by the seedling much more efficiently when it enters through the root system and is transported upwards than when it is introduced through the shoot and transported downwards. This is surprising, since the latter mode of translocation seems to correspond more closely to the normal way of distributing thiamin in the plant.

C. *The effect of pyridoxin*

Repeated experiments with pyridoxin demonstrated that the response of the decotylised seedlings to this vitamin varied considerably with the circumstances. Contrary to thiamin, pyridoxin seemed to be influenced in its activity by the presence of certain other metabolites in the medium. The age or the physiological state of the seedlings apparently also played a rôle difficult to elucidate.

The quantities of pyridoxin required for normal growth of the main root are ten to thirty times greater than those of thiamin (Table 4). The average number of laterals as well as the dry weight of shoot and root are also increased by pyridoxin, whereas the growth rate of the laterals is completely unaffected (cf. Expt. 1200, Table 1).

Discussion

Excised pea roots cannot be cultivated continuously unless they are supplied with thiamin and niacin together with a suitable source of carbon, while the whole pea plant does not require any organic supplements at all if it is grown in the light. Under such circumstances the shoot obviously synthesizes the metabolites required by the root. The probably rather complicated nutritional relations between shoot and root have been discussed by Robbins and Bartley Schmidt (1938, p. 722) in connection with his tomato root culture studies: »Why should the top of the tomato plant form thiazole (or vitamin B₁) while the root does not? It would not appear to be a light relationship, since some fungi apparently form vitamin B₁ in the dark. It would not appear to be related necessarily to chlorophyll, since some fungi (which lack chlorophyll) form vitamin B₁ (5).»

The experiments with decotylised pea seedlings do not indicate, however, that the shoot of this plant is more capable of forming thiamin in darkness than the root. Even in the light the synthesis of this vitamin seemed to proceed at a suboptimum rate during the time of the experiment. The optimum concentration required by the shoot is very small and of the same order of magnitude as that required by the root, perhaps somewhat lower (Robbins and Bartley 1937, Bonner 1937, White 1937).

An alternative interpretation would be that the shoot is actually able to form enough thiamin for its own needs, although nothing is transported down to the root during this stage of the development. Against this, however, speaks the fact that the experiment with capillary injection of thiamin into the shoot indicated a certain translocation to the root, and, furthermore, the fact that the shoot is also stimulated by an addition of thiamin to the agar medium.

Contrary to the excised root the main root of the decotylised seedling does not require niacin. This may either be due to the very short duration of the experiment, which does not permit any symptoms of deficiency to appear or the shoot may be capable of forming niacin in amounts sufficient for its own growth and that of the root. However, the amount of niacin available in the seedling does not seem to permit an optimum development of the entire root system, since the growth of the laterals is markedly retarded in a niacin-free medium.

More surprisingly, the decotylised seedling needed pyridoxin (Fries 1955), a vitamin not required by the excised root (Addicott and Bonner 1938). The necessary concentration is ten to thirty times higher than that of thiamin.

Summary

In a sucrose-mineral-salt medium supplemented with arginine, glycine and adenine the two vitamins thiamin and pyridoxin represent the chief limiting chemical factors for decotylised pea seedlings grown in darkness. To support a normal growth rate of the main root 10 $\mu\text{g./l.}$ ($=0.1 \mu\text{g./culture}$) of thiamin and 100 $\mu\text{g./l.}$ ($=1 \mu\text{g./culture}$) of pyridoxin are needed. As regards other features of the growth and development of the seedlings certain quantitative and qualitative differences in the mode of action of the two vitamins and of niacin amide were observed. Even in the light the amount of thiamin added controlled the rate of growth to a certain degree during the time of the experiment.

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References

- Åberg, B.: Physiologische und ökologische Studien über die pflanzliche Photomorphose. — *Symb. Bot. Upsal.* 8, No. 1: 1. 1943.
- Addicott, F. F. & Bonner, J.: Nicotinic acid and the growth of isolated pea roots. — *Science* 88: 577. 1938.
- Bonner, J.: Vitamin B₁ a growth factor for higher plants. — *Science* 85: 183. 1937.
- Fries, N.: Limiting factors in the growth of the pea seedling root. — *Physiol. Plant.* 6: 292. 1953.
- Chemical factors controlling the growth of the decotylised pea seedling. — *Symb. Bot. Upsal.* 13, No. 1: 1. 1954.
- Vitamin requirements of decotylised pea seedlings cultivated in the dark. — *Experientia* 11: 232. 1955.
- Kögl, F. & Haagen-Smit, A. J.: Biotin und Aneurin als Phytohormone. Ein Beitrag zur Physiologie der Keimung. — *Zeitschr. f. physiol. Chemie* 243: 209. 1936.
- Robbins, W. J. & Bartley, M. A.: Vitamin B₁ and the growth of excised tomato roots. — *Science* 85: 246. 1937.
- Robbins, W. J. & Bartley Schmidt, M. A.: Growth of excised roots of the tomato. — *Bot. Gaz.* 99: 671. 1938.
- Saubert-v. Hausen, S.: On the role of growth substances in higher plants. — *Physiol. Plant.* 1: 85. 1948.
- White, P. R.: Vitamin B₁ in the nutrition of excised tomato roots. — *Plant Physiol.* 12: 803. 1937.

Cytochrome Oxidase Participation in Photosynthetic Fixation of Carbon Dioxide: Specific Light Reversal of Carbon Monoxide Inhibition

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The inhibition of photosynthetic carbon dioxide fixation caused by exposure of barley leaves to nitrogen in light or darkness, to carbon monoxide in darkness, the reversal of nitrogen inhibition by short exposures to air in darkness, and the prevention of the carbon monoxide inhibition by illumination during exposure to the inhibitor have all been interpreted as evidence for the participation of cytochrome oxidase in reactions leading to carbon dioxide fixation (Krall and Burris, 1954). Earlier results that may be interpreted in this same manner are the reversible inhibition of carbon dioxide uptake by anaerobiosis reported by Willstätter and Stoll (1918), the cyanide inhibition of oxygen evolution found by Warburg (1919), and a prolonged lag in resumption of oxygen evolution upon illumination after a period of dark anaerobiosis (Franck et al., 1945; Hill and Whittingham, 1953). Gaffron observed a light-reversible carbon monoxide inhibition of carbon dioxide fixation (1935) which he later attributed to inhibition of a hydrogenase associated with the photoreduction process (Gaffron, 1942-43). Only Hill and Whittingham (1953) concluded that the inhibition they observed could have been the result of a lack of oxidative action during and following the dark period.

This paper reports work which lends strong support to the idea that cytochrome oxidase is an integral part of the photosynthetic machinery.

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Materials and Methods

Cut first leaves of barley (*Hordeum vulgare* var. Oderbrucker) 7—12 days old were used for all the experiments reported here. Second and third leaves of barley, in equal weights, have been used in a few experiments and they respond in the same manner to changes in conditions as do first leaves. The rate of C^{14} fixation was determined with apparatus similar to that described earlier by Krall and Burris (1954) with the exception that $C^{14}O_2$ was not generated each time from $BaC^{14}O_3$ but was made up in large quantities — 20 to 30 ml. — and aliquoted by a gas burette into a vessel similar to that used previously for $C^{14}O_2$ generation. In some cases the exposure to label was started and stopped by solenoid valves controlled by a delay circuit (Krall, 1955). The solenoids used here were type 6 PPW made by the Allen-Bradley Company (of Milwaukee, Wisconsin, USA), to which were attached springs and a blade and anvil, which closed a rubber tubing when the solenoid was not energized.

The light meter used, a Weston Model 756 with a Viscor filter, was calibrated for absolute energy measurement of the sodium lamp emission against a National Bureau of Standards primary standard lamp, a thermopile being used as the energy sensing element. The energy levels reported are accurate to about $\pm 5\%$.

Experiments and Results

The work reported previously (Krall and Burris, 1954) did not establish the light reversibility of the carbon monoxide inhibition; rather, it showed only light prevention of this inhibition; nor were experiments conducted to show the effect of darkness on the plant as differentiated from that of anaerobicity. Such experiments have now been done and are reported in table 1.

The first experiment is the control: it has previously been shown (Krall and Burris, 1954) that the rate of fixation of $C^{14}O_2$ does not vary more than 5 per cent in different batches of barley leaves on the same day. The second experiment shows that exposure to higher concentrations of CO_2 neither increases nor decreases subsequent carbon dioxide fixing ability of the leaves. Since the inhibitions observed with nitrogen were thought to result from prolonged induction effects, rather than from any permanent damage to the plant, it was reasoned that darkness should have some effect on the rate of carbon dioxide fixation as measured here. Experiments 3, 6, and 7 show the magnitude of this effect. The latter two experiments, with 0.1 and 5.0 per cent oxygen in nitrogen, show that only very small partial pressures of oxygen are necessary to prevent complete inhibition, such as those previously observed by Krall and Burris (1954) after incubation with N_2 . Experiments 4 and 5 show the result of exposure of the leaves to 95 per cent carbon monoxide, 5 per cent oxygen mixtures for 45 and 90 minutes. The

Table 1. *Effect of variations of light and of composition of preflush gas on subsequent $C^{14}O_2$ fixation.*

Expt. No.	Pretreatment Conditions			C^{14} fixed (cts/sec/g)	Percentage inhibition
	Flush gas	Min.	Light		
1	air	> 60	2000 fc white	43,000	0
2	air + 1 % CO_2	< 60	2000 fc white	41,400	3.7
3	air	75	Dark	16,200	60
4	95 % CO —5 % O_2	45	Dark	6,360	85.1
5	95 % CO —5 % O_2	90	Dark	2,380	94.5
6	99.9 % N_2 —0.1 % O_2 ..	45	Dark	16,600	61.5
7	95 % N_2 —5 % O_2	100	Dark	14,500	66.0
8 a	95 % CO —5 % O_2	45	Dark		
b	95 % CO —5 % O_2	2	500 fc yellow	13,400	69.0
9 a	95 % CO —5 % O_2	45	Dark		
b	95 % CO —5 % O_2	5	500 fc yellow	27,000	37.0
10 a	95 % CO —5 % O_2	45	Dark		
b	95 % CO —5 % O_2	20	500 fc yellow	28,100	35.0
11 a	95 % N_2 —5 % O_2	45	Dark		
b	95 % N_2 —5 % O_2	5	500 fc yellow	16,600	61.5

One gram of cut barley leaves, 6—7 days old, were preflushed as indicated in column 2. In the last four experiments two conditions were used, a before b. The preflush gas was removed by evacuation and 1 per cent CO_2 in N_2 containing 2.5 per cent C^{14} admitted. The chamber was illuminated by 2000 fc white light during a $C^{14}O_2$ fixation period of 60 seconds. The $C^{14}O_2$ was removed by evacuation and boiling 80 per cent EtOH admitted. An aliquot of the extract was counted in a gas flow proportional counter. Counts are not corrected for geometry.

latter inhibition is greater than that usually obtained with this ratio of inhibitor to oxygen in heart muscle sarcosomes (Slater, 1950). The experiments reported here are typical of several experiments of this type done with barley leaves of different ages. Experiments 8, 9, and 10 show that the inhibition induced by carbon monoxide in darkness is largely reversed by exposure of the tissue, in the presence of inhibiting gas, to yellow light from a sodium arc lamp. The effect of the yellow light is essentially complete within 5 minutes. This compares closely with the time required for the reversal by air of the anaerobic inhibition observed before (Krall and Burris, 1954). Experiment 11 shows that exposure to the yellow light has a greater effect on leaves that have been exposed to carbon monoxide than those where a nitrogen-oxygen mixture was used. It may be that the plant uses carbon dioxide produced by an anaerobic fermentation process induced by the carbon monoxide, which then allows it to pile up photosynthetic intermediates and lessen the usual dark to light induction effect. Support for this idea comes from the fact that inhibition was not as complete in those experiments where the 1 per cent carbon dioxide present in the commercial carbon monoxide used was not removed by an alkali trap.

Table 2. *Effect of red and yellow lights on steady state photosynthesis and reversal of carbon monoxide inhibition of photosynthesis.*

Expt. No.	Pretreatment		Reversal light	cts/sec. $C^{14}O_2$ fixed
	Gas	Time (min.)		
1	1 % CO_2 in air	20	yellow	2,540
2	1 % CO_2 in air	20	red	7,500
3	95 % CO —5 % O_2	60	dark	700
4	95 % CO —5 % O_2	60	2 min, 45 sec red	11,200
5	95 % CO —5 % O_2	60	2 min, 45 sec yellow	19,000
6	95 % CO —5 % O_2	60	10 min red	19,800
7	95 % CO —5 % O_2	60	10 min yellow	22,320

One gram of barley leaves were pretreated as shown and then exposed to the reversal light in the same gas mixture. One per cent CO_2 in N_2 containing about 2 per cent C^{14} was flushed in after evacuating the exposure chamber. The exposure to $C^{14}O_2$ was 45 seconds. The reversal lights and exposure light intensities were about 75 fc for reversal and 2000 fc white for $C^{14}O_2$ exposure. All lights and apparatus were left in the same position throughout the experiments. Yellow lamp was sodium arc (589 m μ); red was from 150-watt reflector spot with red filter (Corning 2030) cut off at 640 m μ . Killing and counting procedures are the same as in table 1.

Utilization of light by photosynthesizing wheat leaves is most efficient in the blue (ca. 450 m μ) and in the red (ca. 660 m μ) regions of the spectrum (Burns, 1937). Warburg and Negelein (1929) have shown that the action spectrum of the photodissociation of a cytochrome oxidase-carbon monoxide complex, which is enzymically inactive, has peaks at 440 and 590 m μ . Hence it was thought possible that the use of red light of a wave length longer than 640 m μ and of light from a sodium vapor lamp (589 m μ) might provide a means of differentiation between a reversal of the carbon monoxide inhibition by direct action of the light on the complex or the indirect effect of competitive reversal by oxygen or other oxidizing material produced by the photolysis of water. The first two experiments of table 2 show the rate of steady-state photosynthesis under the two lights. These experiments were run by substituting $C^{14}O_2$ for the unlabeled gas after 20 minutes of illumination, then killing the leaves with hot ethanol after 45 seconds. The red light was three times as effective as the yellow light in promoting steady-state photosynthesis. The next five experiments (3 to 7) show the amount of inhibition induced, in this series of runs, by a 1-hour exposure to the carbon monoxide, and the differential reversal of this inhibition by either red or the yellow light. The red light was only about half as effective in reversal as was the yellow light at the shorter time. The yellow light had no further effect after 5 minutes as shown by data in table 1, but red light caused an increase in the rate of uptake of $C^{14}O_2$ up until about 20 minutes. The rapid action of yellow light is consistent with the hypothesis that its primary effect

is photodissociation of a carbon monoxide-cytochrome oxidase complex. The slower effect of the red light is probably the result of a gradual buildup of oxygen inside the tissue which displaces the carbon monoxide from the enzyme.

An attempt was made to observe a direct stimulation by oxygen of steady-state photosynthetic carbon dioxide fixation. Barley leaves were exposed, under constant illumination, to nitrogen-carbon dioxide mixtures circulated in a closed system for 20 minutes. They were then allowed to fix labeled carbon dioxide for a short time and the rate of uptake of the label determined by extraction and counting. Light intensity was varied from 50 to 4000 fc and oxygen tension from zero to 20 per cent. No significant stimulation of the rate of $C^{14}O_2$ uptake by oxygen was observed under any of these conditions.

Discussion

Reversibility of a carbon monoxide inhibition of cytochrome oxidase by light of a specific wave length has been termed (James, 1953) the most «elegantly diagnostic» test available to the physiologist seeking in situ confirmation of an enzyme's activity. It has been shown here that preincubation of barley leaves does induce an inhibition and that this inhibition is more rapidly reversed by yellow light than by red light. Thus cytochrome oxidase activity seems to be necessary at least for recovery from the dark-induced carbon monoxide inhibitions observed in these experiments.

The relative effectiveness of the red and yellow lights in steady-state photosynthesis (P_R and P_Y) divided by the effectiveness of these same lights in reversal of the carbon monoxide inhibition (R_R and R_Y) i.e.,

$$\frac{P_R/P_Y}{R_R/R_Y} = Q_E$$

gives a quotient (Q_E) which is a measure of the specificity of the yellow light for reversal of the carbon monoxide inhibition and of the red light for photosynthetic action. If neither light were specific for either process the quotient would be one; if both lights were absolutely specific for the above associated processes it would be infinity. The values in these experiments ran between five for the short time reversals recorded in table 2 down to about two for long time experiments done at 1000 and 2000 fc of red and yellow lights. Thus the yellow light was always somewhat more efficient in photodissociation of the carbon monoxide-cytochrome oxidase complex than was the red light. Its effectiveness became more pronounced at the lower light intensities and at the shorter reversal times. This was to have been

expected if the yellow light promoted photodissociation of an inhibitor enzyme complex, which is known to be instantaneous (Slater, 1950), while the red light brought about a relief of the inhibition by a competitive replacement of the carbon monoxide by internally generated oxygen during slow, autocatalytic recovery from the inhibition. The greater efficacy of the yellow light at the lower intensities probably means that it was effective in relieving the carbon monoxide inhibition at lower intensities than those at which the red light was active in promoting photosynthesis. Only a slight increase in rate of action of the yellow light was seen on increasing the intensity from 50 to 1000 fc. In measuring the light sensitivity of the carbon monoxide complex of the yeast respiratory enzyme, Warburg (1949) found that respiration was doubled in the presence of carbon monoxide when 0.65×10^{-4} cal./cm² min. of blue light illuminated the inhibited suspension. The carbon monoxide-enzyme complex had an optical density, in the blue, 11.5 times as high as in the yellow α band, where the yellow light used here is effective. The 75 fc of yellow light in this system was equivalent to 107×10^{-4} cal./cm² min., which should be sufficient to completely dissociate any enzyme-inhibitor complex. Thus the lack of further effect on increasing the yellow light from 75 to 1000 fc is readily explainable. Photosynthetic rates increase linearly with light intensity up to about 2000 fc in wheat. Barley leaves probably behave in the same manner. Thus any effect which saturates at 75 fc cannot be the result of photosynthetic action. Furthermore, if one were to argue that the slow action of the red light is the result of light effective in reversal of carbon monoxide inhibition which is not removed by the red filter, the argument advanced here that reversal by the yellow light is the result of restored cytochrome oxidase activity is strengthened rather than weakened.

The lack of stimulation of photosynthesis by externally supplied oxygen is in accord with the results of Allen (1955) and of Brown (1953). The enzyme inhibited by carbon monoxide seems to have a very high affinity for oxygen or is so situated in the plant that it receives molecular oxygen directly, perhaps in the form of a complex, from the site of its evolution within the plant. From the results reported here, it can be seen however, that if such a complex exists, it is not a better competitor with carbon monoxide for a site on the enzyme than is molecular oxygen.

The oxygen level necessary for half-maximal rate of oxygen uptake by a number of bacteria known to utilize cytochrome oxidase as a terminal respiratory enzyme has been measured by a polarographic method (Longmuir, 1954). The smallest of these, in which concentration gradients inside the cell are presumably the lowest, had a K_m value at 20° C of 1.1×10^{-8} M oxygen. This corresponds to a partial pressure of oxygen of 0.01 mm of Hg

in a gas in equilibrium with the liquid. Allen (1955) has measured rates of photosynthesis where, under his best conditions, he has maintained oxygen tensions of 0.004 mm of Hg in the liquid. If the possibility is admissible that concentration gradients of oxygen within the cell, or across the cell wall, builds up the oxygen tension inside the cell still higher than in the liquid, it is easily conceivable that an oxygen-requiring reaction mediated by cytochrome oxidase may take place within the cells even under the »extreme anaerobiosis» utilized by Allen. Since the system behaves normally with respect to carbon monoxide inhibition, it is also possible, although not probable that barley cytochrome oxidase may have a higher affinity for oxygen than the bacterial enzyme. This can be determined only by a direct measure on the isolated enzyme using natural reductants.

It must be emphasized that all work so far reported has not shown the participation of oxygen from the atmosphere in steady-state photosynthesis. Brown's measurements (1953) with the recording mass spectrometer showed no stimulation of uptake of external oxygen by light. It would thus seem possible that cytochrome oxidase is necessary only in the dark in order to maintain a normal level of phosphate bond energy, whereas some other process (Frenkel, 1954; Arnon et al., 1954 a, b) might take over generation of high energy phosphate bonds on illumination of the plant. The only experiment which so far speaks against this concept is the inhibition which can be obtained with nitrogen flushing of the leaves under illumination (Krall and Burris, 1954), which indicates that the oxygen, which must be liberated in photosynthetic uptake of internally generated carbon dioxide, was present as the free gas and was swept away by the nitrogen before being utilized again by an oxidase which is spatially removed from the site of oxygen release.

Summary

The inhibition of photosynthetic carbon dioxide fixation which is induced in the dark by exposure to carbon monoxide has been shown to be light reversible. Yellow light, low in photosynthetic activity, promoted the reversal more rapidly than did red light, which is efficient in promotion of steady-state photosynthesis. Thus cytochrome oxidase is active in the photosynthesizing leaf during recovery from the inhibition.

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References

- Allen, F. L.: Observations on photosynthesis and related systems. I. Influence of anaerobiosis on photosynthetic rates during continuous irradiation. — *Arch. Biochem. and Biophys.* 55: 38. 1955.
- Arnon, D. I., Allen, M. B., & Whatley, F. R.: Photosynthesis by isolated chloroplasts. — *Nature (Lond.)* 174: 394. 1954 a.
- Whatley, F. R., & Allen, M. B.: Photosynthesis by isolated chloroplasts. II. Photosynthetic phosphorylation, the conversion of light into phosphate bond energy. — *J. Am. Chem. Soc.* 76: 6324. 1954 b.
- Brown, A. H.: The effects of light on respiration using isotopically enriched oxygen. — *Am. J. Bot.* 40: 719. 1953.
- Burns, G. R.: Photosynthesis and the absorption spectra of plant pigments. I. — *Am. J. Bot.* 24: 257. 1937.
- Franck, J., Pringsheim, P., & Lad, D. T.: Oxygen production by anaerobic photosynthesis of algae measured by a new micromethod. — *Arch. Biochem.* 7: 103. 1945.
- Frenkel, A.: Light induced phosphorylation by cell-free preparations of photosynthetic bacteria. — *J. Am. Chem. Soc.* 76: 5568. 1954.
- Gaffron, H.: Über die Unabhängigkeit der Kohlensäure-assimilation der grünen pflanzen von der Anwesenheit kleiner Säurestoffmengen und über eine reversible Hemmung der Assimilation durch Kohlenoxyd. — *Biochem. Z.* 280: 337. 1935.
- The effect of specific poisons upon the photo-reduction with hydrogen in green algae. — *J. Gen. Physiology.* 26: 195. 1942—43.
- Hill, R. & Whittingham, C. P.: The induction phase of photosynthesis in *Chlorella* determined by a spectroscopic method. — *New Phytol.* 52: 133. 1953.
- James, W. O.: The use of respiratory inhibitors. — *Ann. Rev. Plant Physiol.* 4: 59. 1953.
- Krall, A. R. & Burris, R. H.: Evidence for the participation of cytochrome oxidase in photosynthetic fixation of carbon dioxide. — *Physiol. Planta* 7: 768. 1954.
- Krall, A. R.: A device for steady state photosynthetic carbon dioxide fixation for very short times. — *Plant Physiol.* In Press, 1955.
- Longmuir, I. S.: Respiration rate of bacteria as a function of oxygen concentration. — *Biochem. J.* 57: 81. 1954.
- Slater, E. C.: The components of the dihydrocozymase oxidase system. — *Biochem. J.* 46: 484. 1950.
- Warburg, O.: Über die Geschwindigkeit der photochemischen Kohlensäurezersetzung in lebenden Zellen. — *Biochem. Z.* 100: 230. 1919.
- & Negelein, E.: Über das Absorptionsspektrum des Atmungsferments. — *Biochem. Z.* 214: 64. 1929.
- Heavy metal prosthetic groups and enzyme action. — Translated by Alexander Lawson. Oxford, Clarendon Press. 1949.
- Willstätter, R. & Stoll, A.: Untersuchungen über die Assimilation der Kohlensäure. — Springer, Berlin. 1918.

Studies on the Respiration of Wheat Infected with Stem Rust and Powdery Mildew

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Introduction

Current interest in the problems of resistance to plant diseases has emphasized the desirability of having more information concerning the factors which may contribute to the development of this property. Augmented respiration being a universal consequence of infection of higher plants, many attempts have been made to correlate this process with the basic problems of plant immunity.

As early as 1912 Bach suggested that the tissue respiration of the host may play a part in protecting plants against infection, oxidizing the toxins produced by the pathogens. In the course of the last years Rubin and his co-workers (1953, 1955) have also been particularly interested in the common problems of resistance and respiration. They found very suggestive evidences for the protective nature of oxidative processes from detailed studies carried out on host-parasite complexes such as potato-Phytophthora, cabbage-Botrytis and citrus-Penicillium. The data available from their experiments indicate that the respiration of resistant varieties is generally more intensive than that of the susceptible ones and the increase in respiration, brought about by infection, is more pronounced in resistant plants.

Fuchs and Kotte (1954) have recently demonstrated that resistance of potato to Phytophthora depends on oxidative mechanisms. In the presence of respiratory inhibitors the resistant varieties lose this property and become susceptible.

In contradiction to the majority of the above mentioned authors, Suchorukov (1952) has come to the conclusion that the parasitically increased respiration of the host favors the development of the fungus. Sempio (1943, 1946, 1950), in a series of interesting papers has studied the ratio of photosynthesis and respiration with

regards to resistance of wheat to powdery mildew and reached the conclusion that high ratios (i.e. more anabolic type of metabolism) may be related to resistance.

It should therefore be realised that the relations are generally much more complicated than formerly believed and it is highly questionable whether general conclusions may be drawn at all from the present experimental findings.

A comparison of the main trends in the past with those prevailing in the more recent works reveals particularly remarkable changes in the basic concepts. It is generally believed that the classical approach in the elucidation of the nature of resistance, i.e. the simple comparison of resistant and susceptible plants from several physiological-chemical aspects, have yielded little information so far in regard to the basic questions. The preponderance of observation indicates that the resistance results from an interaction between host and parasite, the protective mechanisms being activated exclusively and specifically by the pathogens.

It seems definitely established that the possible relations between respiration and resistance cannot be solved without further analysis of the mechanisms responsible for the profound changes in the overall process of respiration in infected tissues, because it may be assumed that the response is characteristic also in qualitative relations.

The present paper is concerned therefore primarily with the qualitative analysis of the parasitically increased respiration of wheat-rust and wheat-powdery mildew host-parasite complexes and various respiratory inhibitors were used for this purpose. The problem will be treated in detail on the basis of experiments carried out on susceptible varieties. However, some experiments are in course also with resistant wheats in order to gain in the future some additional data in regard to the above discussed contradictory opinions about the role played by the oxidative processes in resistance. — At the end some experiments will be described which were carried out in order to isolate an active principle (toxin), the presence of which may be postulated on the basis of analogous cases and indirect evidences.

Materials and Methods

Detached leaves of 8—12 days old wheat seedlings were vacuum-infiltrated with 0.05 *M* KH_2PO_4 (control) or with various respiratory inhibitors dissolved generally in the same buffer. The leaves were then laid on a filter paper sheet in order to evaporate the excess of infiltrated water. The respiration rates of approximately 3×3 mm leaf-sections were followed at 30° C. using standard Warburg respirometer techniques for determination of oxygen consumption. The center well contained 0.2 ml 20 per cent KOH to absorb CO_2 and the main compartment of the flask 100 mg fresh material, suspended in 2 ml 0.05 *M* phosphate buffer (control) or with the same amount of buffer with inhibitor dissolved in it (experimental vessels).

Changes in pH in the range of 4.0—7.0 had very little or no effect on the respira-

tory activity. More acid solutions caused, however, more or less severe injuries. Solutions containing inhibitors were adjusted to the pH at which the inhibitor exerts its maximal efficiency according to the recent literary data (James 1953 b). Manometers were read at $\frac{1}{2}$ hr intervals, using thermobarometer corrections. Each of the tests reported in this paper has been repeated from 4 to 10 times. Q_{O_2} values were calculated as the amount of absorption of gas in microliters per 1 mg air-dry matter per hour.

The experimental plants used in this study were grown under ordinary greenhouse conditions. The experiments were conducted with the highly susceptible spring wheat variety Garnet and with the most intensively cultivated Hungarian varieties (Bánkúti 1201, F 481) and essentially the same results were obtained.

The primary leaves of test seedlings were artificially inoculated by using a suspension of *Puccinia graminis* uredospores. After the incubation time (24 hrs in a chamber of 100 per cent relative humidity) the plants were transferred to greenhouse. Respiratory rates were measured when severe infection was observed on the inoculated leaves, i.e. after the outbreak of large, normal pustules (7—9th day after inoculation).

Experiments with powdery mildews were conducted in separate greenhouses and the experimental plants inoculated by dusting them with the spores of heavily infected individuals.

Results

Experiments with respiratory inhibitors

Aerobic respiration of plants may follow several pathways (see the different terminal oxidases and the direct oxidative pathway). It is generally assumed, however, that in most tissues a great percentage of total respiration (measured by O_2 -uptake) is going through an initial reactionspath which is common to both aerobic and anaerobic respirations. If the glycolysis inhibitors (iodoacetate, fluoride) reduce also the rate of O_2 -consumption (in addition to the inhibition of CO_2 -output), it must be concluded that this earlier common path (Embden-Meyerhof scheme) supplies oxidizable intermediates for the terminal oxidations. There are several possible mechanisms by which glycolytic products may be linked to the known terminal oxidases. From the evidence at present available we may say that the tricarboxylic acid cycle should be regarded as the most important link between glycolysis and oxidation. However, the oxidation of the final products of glycolysis (alcohol, lactic acid) and some of its intermediates (triosephosphate) may proceed through a separate reaction path, mainly by way of ascorbic oxidase.

The use of selective inhibitors is a valuable tool for elucidating the question which of the above mentioned mechanisms is in action. If O_2 -uptake is strongly inhibited by malonate under specific conditions, it must be concluded, that respiration is going through the Krebs cycle. The lack of malonate inhibition would indicate that other pathways are followed.

Starting from the above discussed principles, it seemed reasonable to study the respiration of infected tissues, whether the infection influences only the

Table 1. *Increase in respiration of wheat seedlings infected with parasites.*
Effect of mechanical injuries.

Treatment	QO ₂	Per cent increase in O ₂ -uptake. Control = 100 %
Control	2.9	100
Infected with <i>P. graminis</i>	8.9	307
Infected with <i>E. graminis</i>	5.7	196
Leaves with mechanical injuries ...	3.1	106

rate of O₂-uptake or a more profound qualitative change may also be observed. Some evidences supporting this view have already been presented in a preliminary report (Király and Farkas 1955).

Representative data for wheat plants infected with stem rust and powdery mildew are summarized in Tables 1 and 2. Both of the glycolysis inhibitors deeply reduced the O₂-consumption. The percentage inhibition is approximately the same in infected and healthy tissues. It is strongly indicated therefore, that the common path exists in the wheat and this early part of respiration is not altered by infection.

A more thorough change may, however, be observed when using highly diluted malonate solutions. Strong inhibitions (over 60 to 70 per cent) have been obtained only in the healthy tissues, the respiration of infected leaves being much more resistant to malonate (Table 4).

The reported results strongly support the view that in the infected leaves the respiratory pathways of the Krebs cycle are restricted and new ways are followed. The possible mechanism will be discussed in a later section.

The above results leave little doubt that infections alter the ordinary links between glycolysis and oxidation. The question which arises now is how the pattern of terminal oxidations is changed in the host tissues under the influence of parasites. Although more detailed studies are still in progress, it seems to be justified to give a summary of the results so far achieved.

Powerful inhibition of O₂-uptake has been observed in azide-treated wheat tissues, the percentage inhibition being equal in rusted and healthy leaves. This suggests the participation of metal-containing enzymes in the terminal stages of oxidation. However, contrary to expectations, the respiration of healthy tissues proved to be absolutely cyanide-resistant, which is hardly consistent with the previously mentioned results, obtained with azide. In infected tissues considerable inhibition was incited by the cyanide (Table 2).

Some additional observations were made on the malonate-inhibition to provide support for the specific action of this inhibitor under our experimental conditions.

It may be postulated that if malonate inhibits O₂-consumption because of

Table 2. *Effect of glycolysis- and oxidase-inhibitors on the respiration of healthy and rusted wheat leaves.*

Infiltrated compound (M)	QO ₂		Percentage inhibition	
	Healthy	Infected	Healthy	Infected
KH ₂ PO ₄ 5.10 ⁻²	2.9	8.9	—	—
Iodoacetate 10 ⁻³	1.0	3.1	66	66
NaF 10 ⁻²	1.7	5.7	42	36
NaN ₃ 10 ⁻³	0.5	1.3	82	85
KCN 10 ⁻³	2.9	5.7	0	36

a specific interference with succinate oxidation, there is no reason to suppose that anaerobic production of CO₂ would also be affected since the enzyme succinic dehydrogenase is not involved in the latter process. The inhibition of aerobic respiration by malonate causes a »partial anaerobiosis» in the tissues and a shift (rise) of the R.Q. may be anticipated if the effect is specific for the aerobic processes and malonate does not influence the anaerobic CO₂-output. As pointed out by Beevers (1952), uninfluenced CO₂-output in the presence of malonate may be obtained only at rather low concentrations, which are certainly lower than those generally applied in most of the preceding works (0.05 M).

In Machlis's (1944) experiments with barley roots malonate indeed inhibited oxygen consumption and CO₂-production equally. The specificity of inhibitor-action in his experiments has therefore been called in question.

In our experiments, however, with both healthy and rusted leaves it has been found that the application of dilute (0.01 M) malonate solutions strongly reducing oxygen consumption, does not inhibit in a corresponding degree the CO₂-production and the R.Q. rises considerably (from around 1.0 to about 2.1; see Table 3).

Another demonstration of specificity might be the removal or partial removal of malonate inhibition by intermediates of the tricarboxylic acid cycle. A representative experiment is shown in Table 4. Malate, added in concentrations equal to those of the malonate, is able to renew oxygen uptake to a considerable degree.

Table 3. *Effect of infection on the respiratory quotient.*

Treatment	QCO ₂	QO ₂	R. Q.
Healthy leaves	2.8	2.7	1.04
Leaves infected with <i>P. graminis</i>	7.8	7.8	1.00
Healthy leaves infiltrated with 10 ⁻² M-malonate	1.3	0.6	2.16

Table 4. *Effect of dehydrogenase-inhibitors on the respiration of healthy and infected¹ wheat leaves.*

Infiltrated compound	QO ₂		Percentage inhibition	
	Healthy	Infected	Healthy	Infected
KH ₂ PO ₄ 5.10 ⁻² M	2.9	8.9	—	—
Malonate 10 ⁻² M	0.9	5.8	70	35
Malonate 10 ⁻² M + malic acid 10 ⁻² M	1.5	—	48	—
Malonate 10 ⁻² M (mildewed leaves).....	0.9	3.4 ²	70	40
Methylene blue 5.10 ⁻⁴ M	3.2	—	0	—
Malachite green 1:5000	2.2	7.7	26	14
Malachite green 1:5000 (mildewed leaves)	2.2	5.1 ²	26	11

¹ If otherwise not noted, infection means rusted plants.

² The data of leaves infected with powdery mildew and infiltrated with K₂PO₄ see on Table 1.

The above results support the view that the initial and terminal stages of respiration are slightly affected by infection. *The main attack is exerted probably on the link between glycolysis and terminal oxidations.* Since these reactions involve dehydrogenations, it was anticipated that their susceptibility to chemical influences may be different in healthy and invaded tissues.

There are claims that the respiratory rates may be activated within the living tissues of several plants with the aid of redox dyes (methylene blue, 2,6-dichlorophenolindophenol) catalyzing hydrogen transfer (James 1953 a). In our special case, however, no significant increase in O₂-uptake has been obtained by means of these catalytic substances.

Nevertheless, the inverse approach of the problem, i.e. the inhibition of dehydrogenations by malachite green, revealed slight but reliable differences in the behaviour of the experimental plants. Significant inhibition has been observed exclusively in the control leaves (Table 4).

Malachite green is known as a dye inhibiting a number of dehydrogenases. Its specificity *in vivo* was but slightly studied and therefore the exact point of attack under these conditions remained unknown up to the present. Without discussing the problem in detail it may be concluded that the results obtained are in good accordance with the view that the parasitic effect is focussed on the link between glycolysis and terminal oxidases and the respective role of different dehydrogenases participating in this process (see the succinic acid dehydrogenase) may be changed.

Experiments on the toxin action

There is today general agreement that the symptoms of parasitic diseases are incited largely by such chemical substances excreted by the fungus, which have

injurious effects upon the host. The presence of toxic substances produced by mildews would be expected on the basis of indirect evidences deriving from observations made on mildewed wheats (Allen 1953).

In some cases the chemical structure of the toxins has also been determined and some of the processes leading to the characteristic effects are now fairly well understood (Uritani et al. 1953, 1954).

In order to gain a deeper insight into the mechanisms by which respiration is stimulated in rust- and mildew-infected wheat seedlings we tried to isolate the active principle responsible for the increased O_2 -uptake. However, these efforts remained unsuccessful up to the present.

Water extracts were made from both mildew- and rust-infected leaves, infiltrated in healthy ones and respiratory rates measured after an incubation period of 4 to 10 hours. The experiments did not reveal any significant differences in the O_2 -consumption of experimental and control leaf pieces (infiltrated with extracts from healthy leaves).

These experiments, however, do not exclude definitely the possibility of a toxin action. The failure may be attributed to the hardly diffusible character of the toxic metabolites, having certainly high molecular weight, or may be explained by the fact that the compound is highly labile and could be destructed and inactivated by the experimental procedures.

Discussion

The observations and experiments reported in this paper establish the fact that the parasitically increased respiration of wheat-rust and wheat-mildew host-parasite complexes follows new pathways. The question now arising is whether the quantitative changes observed in these parasitic complexes may really be explained by host-parasite counteractions and can be attributed to alterations in the metabolic pathways operating in the host tissues. As long as this fact has not been proven, one may consider that the reported quantitative and qualitative changes should be explained by the additional and undistinguishable respiration of fungal hyphae present in host tissues. However, the detailed investigations of several authors have demonstrated that it is not impossible to distinguish between the respiration of host and the parasite (Yarwood 1934, Allen and Goddard 1938, Arzichowskaja 1946, Uritani et al. 1954).

All these results are consistent with the view that the observed changes in the overall respiration must be attributed to the altered metabolism of host tissues.

Although cereal rusts do not appear as well convenient for similar studies to distinguish between the metabolic processes of host and parasites as for example the ectoparasite mildews are, some indirect evidences should be mentioned which indicate that the same relations may be present in rust-infected tissues.

Some of the discussed findings were confirmed with mildew infected wheats as well (see the effect of malonate, Table 4) and the results are similar to those obtained

with rusted plants. Considering that the possible role of the fungus as a decisive factor is already excluded by the studies mentioned on mildews (Allen and Goddard 1938) our results appeared reliable with both mildew- and rust-infected leaves.

In addition it must be referred to the paper of Shu et al. (1954) on the respiratory systems of germinating rust uredospores whose metabolic pattern may throw some light on the respiratory systems of rust mycelia developing inside the plant. According to these investigations the O_2 -uptake of germinating spores is totally insensitive to NaF and partially insensitive to NaN_3 . If the rust respiration were a decisive fraction in the total respiratory increase of parasitized leaves, the diseased leaf sections would exhibit a diminished inhibition by these compounds in comparison to the values obtained with healthy tissues. It has been shown namely that the oxygen uptake of healthy leaves is almost totally depressed by azide and considerably inhibited by fluoride (Table 2). The percentage inhibition would therefore be less in infected tissues if the fluoride- and azide-resistant respiratory systems of rust were responsible for the additional respiration. But in the contrary the degree of both azide- and fluoride-inhibition is practically the same in healthy and infected tissues (Table 2).

According to the experiments of the Canadian group the R.Q. of the germinating uredospores is diminished to 0.46 by the addition of malonate. The R.Q. of infected leaves treated with malonate is raising over the unity, like the values obtained with malonate-poisoned healthy plants. This would be impossible if rust respiration would play a major role in the gas exchange of the whole complex.

It may also be concluded from the available data that the rust is equipped with a fat-metabolizing system (R.Q. of germinating spores 0.7) while the respiratory substrate of both healthy and rusted leaves is certainly carbohydrate (R.Q. 1.0).

The evidence is therefore convincing that the respiratory changes observed in the wheat-rust and wheat-powdery mildew complexes are actually caused by alterations in the metabolic pattern of the host.

To strengthen this view a second possibility was to be excluded.

Already in the early years of respiratory studies it has been found generally that injuries may exert a stimulatory effect on plant respiration. The typical symptoms of stem rust infection (the epidermis and subepidermal layers are damaged by the developing and outbreking spore masses) necessitated to evaluate the significance of this factor in the overall process of increased gas exchange. Injuries were therefore made with a needle on healthy wheat leaves, comparable to those caused by rust infection and the respiratory rates were compared. The stimulation observed in the injured leaf pieces was insignificant in relation to the respiratory increases measured in infected tissues. The injury effect was thus eliminated as a possible source of respiratory stimulation in rusted plants (Table 1).

The main problem to be discussed in the present section is the possible path of respiration in diseased plants. A theory has been advanced by Allen (1953) concerning the mechanism of these alterations, based mainly on the experiments of Sempio. According to these views in mildew-infected leaves the Pasteur effect (supression of carbohydrate breakdown in air) will be abolished and the aerobic O_2 -consumption thus increased.

A similar case has really been demonstrated by Uritani et al. (1954) on the sweet potato-*Ceratostomella* complex. The toxin produced by the fungus

reduces the efficiency of oxidative phosphorylations associated with electron transfer from substrate to oxygen, i.e. uncouples respiration from the energy-requiring processes, accelerating hereby the O_2 -uptake. This fact would be explicable as due to augmented ATPase activity. Increase in ATPase activity has been made responsible by various authors for the abolition of Pasteur effect (cf. e.g. the review by Turner 1951).

The results obtained by the Japanese group are therefore in agreement with the view outlined above. The question which remains now to be settled is whether our data are also consistent with this theory.

One of the most important facts to be stressed is that according to the presented data (malonate inhibition) the major part of respiration in healthy wheats (grown in greenhouse) follows certainly the tricarboxylic acid cycle. (The respiratory behaviour of wheats grown in the field reveals quite opposite characteristics, their respiration being highly resistant to malonate! A more detailed description of these results will be given shortly by Konrád). However, the degree of malonate inhibition decreases with infection. At the same time the glycolysis inhibitors suppress the O_2 -uptake equally in both groups (healthy and diseased plants) indicating that the initial stages of glycolysis may have an equal significance in the respiration of healthy and infected tissues. The difference may therefore appear in a later stage, i.e. in the mode of oxidation of glycolysis products. If the pyruvic acid is not oxidized via the Krebs cycle but as a result of infection, the whole series of glycolysis reaction-steps is going to completion, the end-products (alcohol, lactic acid) may be oxidized without participation of the tricarboxylic acid cycle.

These ideas, which are consistent with the reported experimental data, are in accord with the view that the Pasteur effect is inhibited by infection, because Pasteur effect means the inhibition of fermentation under aerobic conditions. An aerobic fermentation may therefore be induced in infected plants.

The usual reactionpath of oxidation of the glycolysis endproducts in the plants may be mediated through the ascorbic oxidase (James 1953 a, c). Recently the view has gained ground that ascorbic oxidase plays a decisive role in the later developmental stages of cereals and replaces the cytochrome oxidase operating vigorously in embryos (Goddard and Meeuse 1950, Sys-sakjan *et al.* 1953). According to the presented data the terminal oxidase in wheat plants must have really a metal-containing reactive group and the relative significance of this system in both healthy and diseased plants is nearly the same. This view is substantiated by the results obtained with azide (pH 4.5). A most peculiar feature of our experiments is, however, that the respiration of two weeks old healthy wheat seedlings was completely resistant to the action of cyanide. (In the roots of germinating seedlings a strongly cyanide-

sensitive respiration was found in accordance with the literary data). The result seems to be somewhat surprising, the effect of cyanide and azide being considered generally as identical in the papers dealing with specific inhibitors (Stenlid 1949, James 1953 b).

It must be assumed either that a particular case has been found in which this general rule cannot be applied or — which is more likely — in the wheat some mechanisms must be taken into account which protect the metal-containing enzyme from the cyanide action. In evaluating negative results obtained with respiratory inhibitors the possible role of protecting mechanisms must always be considered.

The considerable cyanide-sensitive fraction of respiration observed in parasitized leaves cannot be attributed to the diminished activity of cyan-resistant flavoprotein enzymes, because the azide-sensitivity persists in infected plants as well. It is more likely that in the infected wheat the mechanism which protects the metal-containing oxidase from the reaction with cyanide will be partially eliminated.

The further investigation of all these problems is in course.

Summary

1. The respiration of wheat seedlings infected with stem rust and powdery mildew is considerably augmented.
2. By the use of various inhibitors it has been found that the parasitically increased respiration involves also qualitative changes in comparison with the normal respiration.
3. The respiration of healthy plants is malonate-sensitive but on the contrary that of the infected tissues is highly insensitive to malonate.
4. The glycolysis inhibitors (fluoride, iodoacetate) are equally influencing the respiration of healthy and diseased plants.
5. The respiration of both control and infected plants is highly sensitive to azide. The O_2 -uptake of healthy tissues is totally resistant to cyanide. However, the infection is correlated with the development of a cyanide-sensitive fraction.
6. R.Q. in both healthy and diseased plants is around 1.0. In malonate treated tissues the R.Q. rises over 2.0.
7. Contrary to expectations, the presence of toxin could not be detected.
8. The results seem to substantiate the fact that the respiratory changes are taking place in the tissues of host and cannot be explained by the additive effect of fungus respiration.
9. An attempt has been made to interpret the parasitically stimulated respiration in biochemical terms. As a theory of the possible mechanisms

the proposed views about the abolishing of Pasteur effect have the greatest possibility at present.

References

- Allen, P. J.: Toxins and tissue respiration. — *Phytopathology*. 43:221. 1953.
 — & Goddard, D. R.: A respiratory study of powdery mildew of wheat. — *Am. Journ. Bot.* 25:613. 1938.
 Arzichovskaja, E. V.: The physiology of *Botrytis-Brassica* complex. — *Mikrobiologija*. 15:47. 1946.
 Bach, A. N.: Collected biochemical papers. — Moskva. 1950.
 Beevers, H.: Malonic acid as an inhibitor of maize root respiration. — *Plant Physiol.* 27:725. 1952.
 Fuchs, W. H. & Kotte, E.: Zur Kenntnis der Resistenz von *Solanum tuberosum* gegen *Phytophthora infestans* de By. — *Naturwiss.* 41:169. 1954.
 Goddard, D. R. & Meeuse, B. J. D.: Respiration of higher plants. — *Ann. Rev. Plant Physiol.* 1:207. 1950.
 James, W. O.: *Plant Respiration*. — Oxford. 1953 a.
 — The use of respiratory inhibitors. — *Ann. Rev. Plant Physiol.* 4:59. 1953 b.
 — The terminal oxidases of plant respiration. — *Biol. Rev.* 28: 245. 1953 c.
 Király, Z. & Farkas, G. L.: Über die parasitogen induzierte Atmungssteigerung beim Weizen. — *Naturwiss.* 42:213. 1955.
 Konrád, É.: Unpublished results.
 Machlis, L.: The influence of some respiratory inhibitors and intermediates on respiration and salt accumulation of excised barley roots. — *Am. Journ. Bot.* 31:189. 1944.
 Rubin, B. A. & Arzichovskaja, E. V.: Biochemische Charakteristik der Widerstandsfähigkeit der Pflanzen gegenüber Mikroorganismen. — Berlin. 1953.
 — Chetverikova, E. P. & Arzichovskaja, E. V.: Oxidative systems and plant immunity. *Žhurn. obsč. biol.* 16:106. 1955.
 Sempio, C.: Influenza dell' oscurità sulla respirazione e sulla glicolisi di alcune piante in rapporto alla loro resistenza a parassiti obbligati. — *Rivista di Biol.* 35:129. 1943.
 — Sulla resistenza metabolica in patologia vegetale. — *Rivista di Biol.* 38:191. 1946.
 — Metabolic resistance to plant diseases. — *Phytopathology*. 40:799. 1950.
 Shu, P., Tanner, K. G. & Ledingham G. A.: Studies on the respiration of resting and germinating uredospores of wheat stem rust. — *Canad. Journ. Bot.* 32:16. 1954.
 Stenlid, G.: Some notes on the effect of sodium azide, 2,4-dinitrophenol, and ortho-phenanthroline upon oxygen consumption in green leaves. — *Physiol. Plant.* 2:61. 1949.
 Suchorukov, K. T.: *Physiology of plant immunity*. — Moskva. 1952.
 Syssakjan, N. M. & Filippovič, I. I.: The characteristics of metabolism and the phasic development of organisms. — *Žhurn. obsč. biol.* 14:215. 1953.
 Turner, J. S.: The Pasteur effect in plants. — *Ann. Rev. Plant Physiol.* 2:145. 1951.
 Uritani, I. & Takita, S.: Studies on the phytopathological chemistry of black rotted Sweet Potato. Part 8. Abnormal increase of the respiration of injured Sweet Potato. — *J. agric. chem. Soc. Japan.* 27:168. 1953.
 — Akazawa, T. & Uritani, M.: Increase of respiratory-rate in Sweet Potato tissue infected with black rot. — *Nature*. 174:1060. 1954.
 Yarwood, C. E.: Effect of mildew and rust infection on dry weight and respiration of excised clover leaflets. — *Journ. Agr. Res.* 49:549. 1934.

Auxin Destruction, Peroxidase Activity, and Peroxide Genesis in the Roots of *Lens culinaris*

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Introduction

It has recently been shown (2) that the ability of pea seedling cells of both root and stem to destroy the native auxin, indoleacetic acid, rises as the cells age. This increased IAA-oxidase activity may be the cause of the decreased sensitivity of older cells to auxin, and may, in fact, constitute a biochemical ageing mechanism. It also appears that the IAA oxidase complex behaves like an induced enzyme, rising in response to increased exogenous auxin levels. Both the peroxide-generating (13) and peroxidase (3, 5) components of the IAA oxidase complex of peas (1) similarly increase in activity in response to exogenous IAA.

In the present work, measurements of IAA oxidase activity, peroxidase activity and peroxide generating capacity have been made in serial sections of the roots of *Lens culinaris*. The growth and auxin relations (7, 8, 9, 10, 12) and physiological gradients (11) in this organ had previously been studied, so that it was possible to relate the biochemical data to developmental processes.

Materials and Methods

Primary roots of the seedlings of *Lens culinaris*, germinated at 23° C. in continuous darkness were used in all experiments. These conditions are

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known (12) to be optimal for growth. Eighteen mm.-long roots were selected because at this stage, rate of elongation is at a maximum (7). For each experiment, 100 similar roots were selected and each root cut into six fragments of 3.0 mm. length.

Determinations of IAA oxidase activity (2), peroxidase activity (3), peroxide genesis (13), and protein nitrogen (2) were made according to previously published techniques or slight modifications thereof.

Results

1. *Fresh weight and protein nitrogen determinations*

Figures 1 and 2 show the distribution of fresh weight and protein nitrogen along the various regions of the root. Protein N was estimated by trichloroacetic acid precipitation of an aliquot of the *brei*, followed by sulfuric acid- H_2O_2 digestion of the precipitate and direct Nesslerization of the resulting ammonia. Fresh weight per section is seen to increase with increasing distance from the tip, while protein nitrogen per unit fresh weight or per section is at a minimum in the region of the meristem, rising sharply toward the root cap, and gradually toward the basal portion of the root.

2. *Indoleacetic acid oxidase*

In vitro IAA oxidase assays were performed by first converting the tissues to a *brei*. The tissues were removed from the medium by decantation onto wire gauze, rinsed with 0.1 M. pH 6.1 phosphate buffer and transferred to a previously chilled mortar containing a little sand and cold buffer. They were then ground and the *brei* subjected to centrifugation (15 min; $3000\times g$). The supernatants were decanted into 10 ml. volumetric flasks and made up to volume with buffer. Aliquots of such breis were incubated with IAA in a metabolic shaking incubator thermostated at 30°C . Initial (5×10^{-4} M) and residual IAA concentrations at various times were determined by the Sal-kowski colorimetric technique (14) on a 2 ml. aliquot.

Data on IAA-oxidase activity, measured as μg IAA destroyed after 60 min., are plotted in Figure 3. They indicate that IAA oxidase activity is low on the region of the meristem and increases in both directions (root cap and base) toward the older cells. A further localization of IAA oxidase activity in the first segment was performed by separating the major portion of the root cap (0—0.5 mm.) from the subjacent tissues (0.5—3.0 mm.). Results expressed in Table 1 indicate a large increase of IAA oxidase activity in the root cap, over and above the low level in the region of the younger tissues.

IAA oxidase activities were also determined in the presence of 2,4-dichlorophenol (DCP), a compound which has been shown to promote IAA oxidase

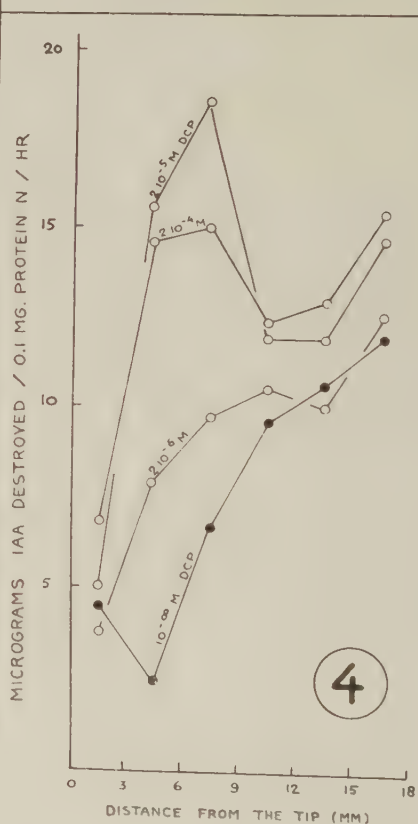
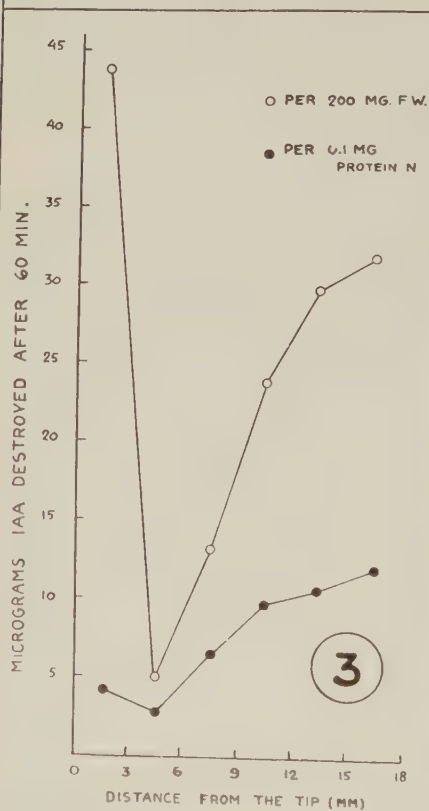
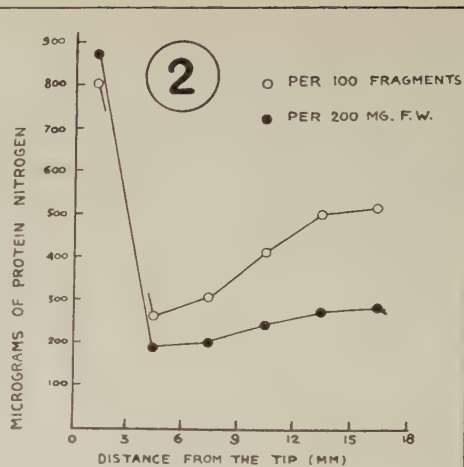
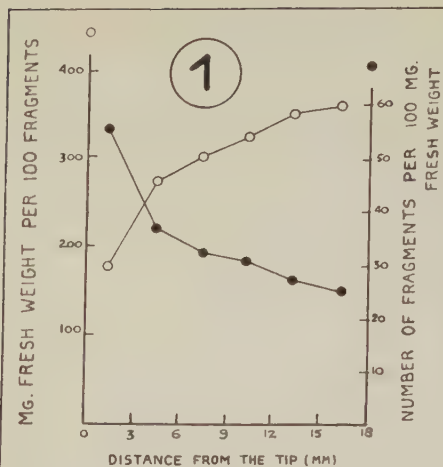


Table 1. *IAA-oxidase activity in the »root cap» and »meristematic region» and the effect of DCP on each region.*

Region	0 to 0.5 mm. tip (± 0.1 mm)		0.5 to 3.0 mm. region (± 0.4 mm.)	
DCP (<i>M</i>)	0	2.10^{-5}	0	2.10^{-5}
Fr. wt. (mg) per 100 fragments	28.4	26.5	163.7	164.0
μ g IAA destroyed/hr per 100 fragments	19	21	26	74
per 100 mg fr. wt.	67	79	16	45
Enhancement by DCP (fr. wt. data)	—	18 %	—	182 %

Table 2. *The distribution of IAA oxidase activity along the root and the effect of DCP on each region.*

Distance from the tip (mm.)	IAA oxidase activity (μ g IAA destroyed/100 μ g protein N/hr.)						
	0 DCP	2.10^{-6} <i>M</i> DCP		2.10^{-5} <i>M</i> DCP		2.10^{-4} <i>M</i> DCP	
	Activity	Activity	Enhancement by DCP %	Activity	Enhancement by DCP %	Activity	Enhancement by DCP %
0—3	4.5	4.0	— 11	5.0	11	6.5	44
3—6	3.0	7.4	150	15.5	416	14.0	366
6—9	6.5	9.5	46	18.5	184	15.0	130
9—12	9.5	10.5	10	12.5	21	12.0	27
12—15	10.5	10.0	— 5	12.5	9	13.0	24
15—18	11.5	12.0	4	14.5	26	15.5	34

activity (4) and peroxide genesis (13) in the pea and the oxidation of IAA by pure horseradish root peroxidase (6). The results, depicted in Figure 4, show that the addition of DCP increases IAA destruction all along the root. However, the region of the young tissues shows greatest enhancement and the root cap and basal regions the least. In table 2, it is shown that a DCP concentration of about 2×10^{-5} *M* is optimal for the young tissues, in agreement with previous findings for the pea (4). A further refinement of the localization in the 0—3.0 mm. fragment was performed by again separating the apical 0.5 mm. from the remaining 2.5 mm. and measuring the effect of DCP on each subfragment. The results of table 1 show a negligible enhancement of activity by DCP in the root cap, and a marked enhancement in the subjacent region.

Figure 1. *Mg* fresh wt. per 100 fragments (○) and number of fragments per 100 mg fresh wt. (●) expressed as a function of distance of the fragment from the root tip.

Figure 2. Protein N content of the various root fragments.

Figure 3. *In vitro* IAA-oxidase activity of the various root fragments.

Figure 4. The effect of various concentrations of 2,4-dichlorophenol (DCP) on the *in vitro* IAA oxidase activity of *Lens* roots.

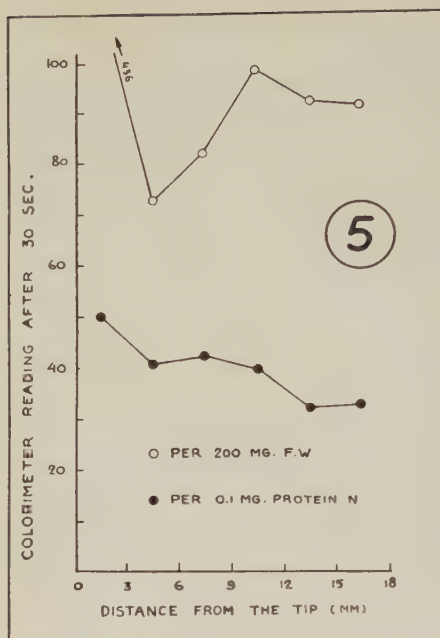


Figure 5. *In vitro* peroxidase activity of the various root fragments.

3. Peroxidase

Aliquots of the *brei* were employed for assay of peroxidase activity, following previously described techniques (3). At zero time, 0.1 ml. of 0.5 *M* pyrogallol was blown vigorously into a Klett colorimeter tube containing 9.9 ml. of *brei*-buffer- H_2O_2 . The formation of purpurogallin was followed colorimetrically for 30 seconds, during which time the reaction proceeds linearly.

Representative data are shown in figure 5. It can be seen that unlike IAA oxidase, peroxidase activity per unit protein decreases gradually but steadily from the tip to the base. This indicates that the great differences in IAA oxidase activity of the various regions are not attributable to differences in peroxidase.

Table 3. *Peroxidase activity of »root cap» and »meristematic region».* The peroxidase unit used here is Klett color developed in 30 sec. with 5.10^{-3} *M* pyrogallol as substrate and 1 ml. of *brei* per 10 ml. reaction mixture.

Region	»Root cap» (0—0.5 mm)	»Meristematic region» (0.5—3.0 mm)
Fresh wt. (mg) per 100 fragments	28.5	160.4
Peroxidase activity per 100 mg. fr. wt.....	112	261

Table 4. *Effect of DCP concentration on peroxide formation expressed in μ moles purpurogallin per 200 mg fresh weight.*

Distance from the tip (mm)	Concentrations of DCP (M)				
	10 ⁻⁸	1.10 ⁻⁵		1.10 ⁻⁴	
	Purpurogallin value	Purpurogallin value	% increase	Purpurogallin value	% increase
0—3	391	442	13	482	23
3—6	174	329	89	440	152
6—9	190	312	64	394	107
9—12	282	360	27	410	45
12—15	300	353	17	412	37
15—18	308	332	7	371	20

As in the previous experiments, a further localization was attempted by separating the apical 0.5 mm. from the subjacent 2.5 mm. Here it was found (Table 3) that peroxidase activity is high in the meristem, and low in the root cap. Thus it appears that peroxidase activity per cell, unlike IAA oxidase, declines as the cell ages.

4. Peroxide Genesis

Peroxide genesis was measured, as previously described (13), by incubating intact tissue with pyrogallol in buffer in the absence of exogenous H₂O₂. Under such conditions, conversion to purpurogallin is limited by production of H₂O₂ in the tissue. Thus, a colorimetric measurement of the amount of purpurogallin formed permits a calculation of peroxide genesis by the tissue.

One hundred roots were harvested as before, cut into successive 3-mm. sections, and placed in 10 ml. of 0.1 M pH 6.1 phosphate buffer.

The formation of purpurogallin began promptly on the addition of pyrogallol to the medium and maintained an approximately linear course for the entire 12-hour period of the experiment (figure 6). DCP increased the rate of peroxide genesis, but as in previous *in vivo* experiments (4) 10⁻⁴ M was more effective than 10⁻⁵ M. Tissues handled under a weak green safelight in a physiological darkroom (figure 6 a) showed a lower initial rate of peroxide formation than those exposed to laboratory light (figure 6 b), the differences between the two treatments diminishing with time. Essentially, then, there are no differences between peroxide genesis in Lens roots and in the various tissues of the etiolated pea plant.

Peroxide genesis was also studied as a function of position on the root. The results shown in Figure 7 indicate that peroxide formation per unit fresh weight is low in the region of the young tissues and increases sharply

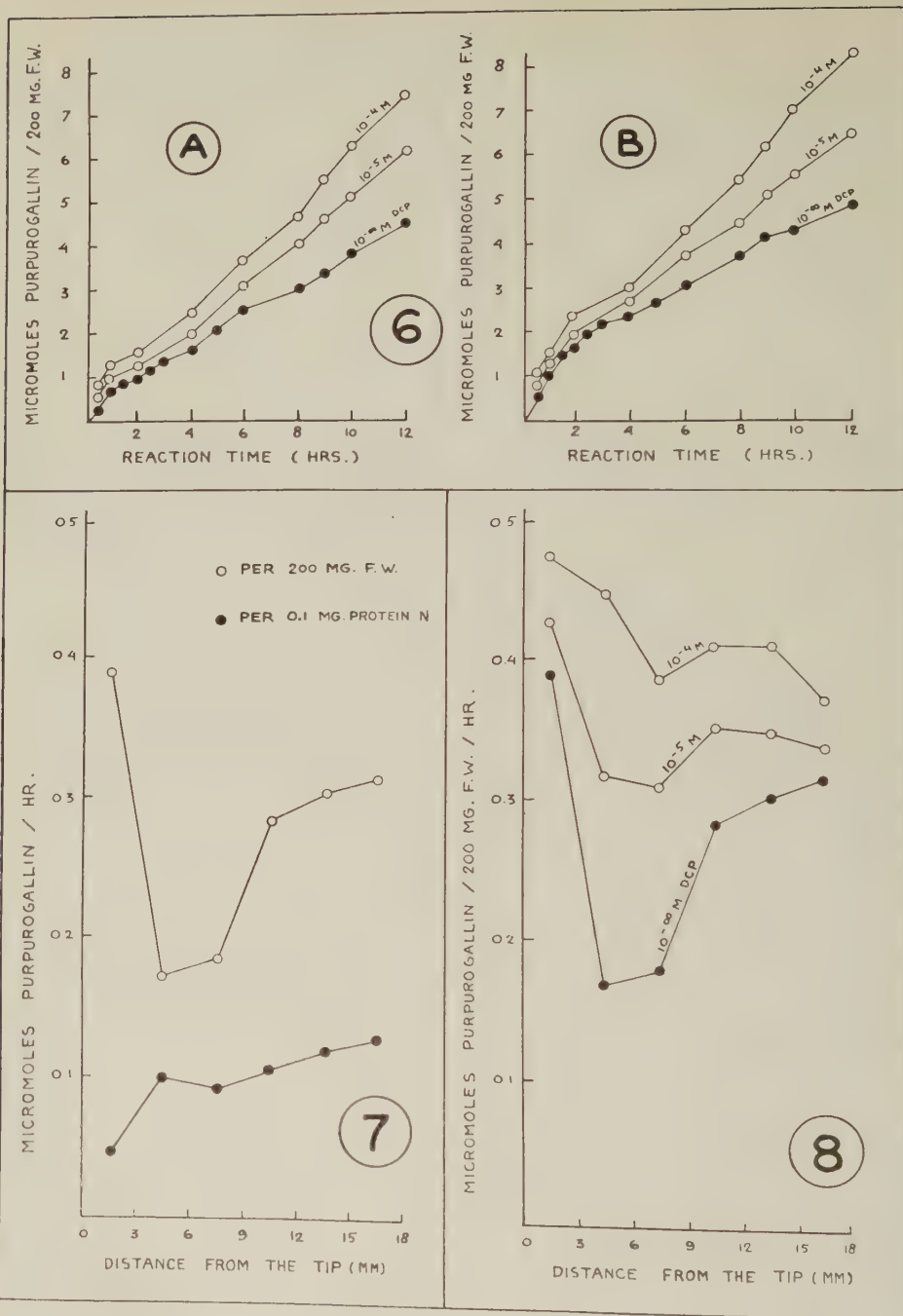


Table 5. *Peroxide genesis in »root cap» and »meristematic region» as affected by DCP and IAA.*

Property determined	»Root Cap» 0—0.5 mm			Meristematic region (0.5—3.0 mm)		
	Control	10 ⁻⁴ M DCP	10 ⁻⁶ M IAA	Control	10 ⁻⁴ M DCP	10 ⁻⁶ M IAA
Fresh wt. (mg)..... per 100 fragments	27.3	28.0	29.9	162.8	164.1	163.0
m μ M purpurogallin per 100 fragments	165	171	183	194	336	299
per 100 mg. fr. wt....	604	610	612	119	204	183
Enhancement %	—	0.9	1.3	—	71.4	53.8

in both directions (root cap and base) toward the older cells. When the results are expressed per unit protein, however, it is seen that the region of the root cap is lower in peroxide generating capacity than all the other regions.

The addition of DCP to the reaction mixture results in a marked increase in peroxide genesis, the increase being greater the greater the DCP concentration added. (Figure 8). As with IAA oxidase, the DCP enhancement of activity is greatest in the meristematic region and young tissues, and least at the root cap and basal regions of the root. (Table 4). Subdivision of the apical 3.0 mm. segment into root cap and subjacent tissues revealed that the former is insensitive to DCP, but peroxide genesis in the latter is greatly enhanced by both DCP and IAA (Table 5).

Discussion

The findings of this paper support and extend those of previous publications. In the root of *Lens*, as in root and stem of *Pisum* (2) and root of *Vicia* (5) it appears to be true that the ability of cells to destroy indoleacetic acid increases with increasing age.

The significance of the IAA oxidase activity in the various regions of the root is best judged by its close correlation with auxin distribution. It has been shown in the root of *Lens* (7) that auxin content is greatest in the region of the young cells, and decreases the further one goes from the meristematic region. Since this pattern is just the reverse of the IAA oxidase distribution.

Figure 6. *In vivo* peroxide genesis in roots kept in the dark (A) and light (B).

Figure 7. *In vivo* peroxide genesis as a function of position on the root.

Figure 8. The effect of various concentrations of DCP on *in vivo* peroxide genesis in the various root fragments.

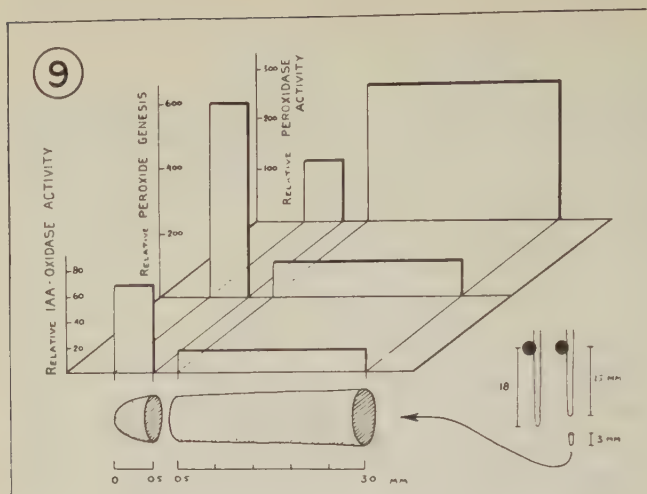


Figure 9. A comparison of the IAA-oxidase, peroxide-geneses, and peroxidase activities of the root cap (apical 0.5 mm) and meristematic region (next 2.5 mm) of the *Lens* root.

the suggestion can be made that IAA oxidase *determines* the endogenous auxin level, high oxidase meaning low auxin, and *vice versa*.

It is clear that the gradient in IAA-oxidase activity is not caused by a gradient in its peroxidase component: rather, it seems to be correlated with peroxide producing capacity (Figure 9). From this correlation, and from the parallel enhancement of IAA-oxidase activity and peroxide generating capacity of the meristematic region by DCP, one may draw the conclusion that overall peroxide genesis and auxin destruction are limited by a DCP-like component in the tissue. This DCP-like component would be the true «ageing factor». Experimental techniques for testing this hypothesis are at hand.

Also of interest is the fact that IAA itself causes an increased peroxide genesis (Table 5). This gives support to other reports of this effect (13, 15), and to the suggestion that IAA, like dioxymaleic acid, gives rise to the peroxide involved in its own peroxidation. With pea tissue, prolonged starvation was required before the peroxigenic action of IAA was manifest, but with the young region of the *Lens* root, the effect can be demonstrated on fresh tissue.

Exposure of tissue to light produces a marked rise in peroxide production. This effect, also previously reported in *Pisum* (13), suggests that the enhanced auxin destruction which is known to result from illumination may be an indirect effect of increased peroxide genesis. It also suggests that some of the morphogenetic effects of light may be channeled through the peroxide — IAA oxidase — auxin destruction pathway.

A recent article by Lockhart (6) reports that DCP is a necessary cofactor for the peroxidation of IAA by horseradish root peroxidase, even in the

presence of excess H_2O_2 . This raises the possibility that what we have called »peroxide genesis» effects may actually be »peroxidase cofactor» effects. This does not, however, materially change the physiological conclusions drawn in the paper.

Summary

The young root of *Lens culinaris*, previously investigated as to growth, auxin relations and physiological gradients, has in this work been examined for various biochemical characteristics. In corroboration of previous work with *Pisum* and *Vicia* it has been found that the capacity of root cells to destroy the native auxin, indoleacetic acid, increases with increasing age. This increased IAA-oxidase activity of the older cells is correlated with, and apparently due to, an increased peroxide-generating capacity. It is not correlated with peroxidase activity, which declines from meristem to older cells.

2,4-dichlorophenol (DCP), applied at a concentration of 10^{-4} to 10^{-5} M, produces a dramatic and parallel increase in peroxide generating capacity and IAA oxidase activity in young cells, but not in old cells. This implies that some naturally-occurring DCP-like factor increases in concentration from young to old cells; the increased concentration of this factor enhances peroxide genesis and therefore auxin destruction. Such a factor could be responsible for the ageing of cells, at least in the sense of decreased ability to grow in the presence of indoleacetic acid.

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References

1. Galston, A. W., Bonner, J. & Baker, R. S.: Flavoprotein and peroxidase as components of the indoleacetic acid oxidase of peas. — Arch. Biochem. & Biophys. 42: 456. 1953.
2. Galston, A. W. & Dalberg, L.: The adaptive formation and physiological significance of indoleacetic acid oxidase. — Amer. Jour. Bot. 41: 373. 1954.
3. — Some metabolic consequences of the administration of indoleacetic acid to plant cells. — Proc. 3rd Intl. Plant Growth Subst. Symposium, Wye College, England. 1955.
4. Goldacre, P. L., Galston, A. W. & Weintraub, R. L.: The effect of substituted phenols on the indoleacetic acid oxidase activity of etiolated peas. — Arch. Biochem. & Biophys. 43: 358. 1953.
5. Jensen, W.: The induction of peroxidase activity by indoleacetic acid. — (Plant Physiol., submitted for publication).
6. Lockhart, J.: The role of 2,4-dichlorophenol in the destruction of indoleacetic acid by peroxidase. — Plant Physiol. 30: 86. 1955.
7. Pilet, P. E.: Contribution à l'étude des hormones de croissance dans la racine du *Lens culinaris*. — Mem. Soc. vaud. Sci. nat. 64: 137. 1951.

8. — Répartition et variations des auxins dans les racines du *Lens culinaris*. — *Experientia* VII/7: 362. 1951.
9. — Physiologie des racines du *Lens culinaris* et hormones de croissance. — *Phyton* (Graz) 4: 247. 1953.
10. — Croissance et rhizogenèse des racines de plantules vernalisées et rôle du froid sur les auxines et leurs précurseurs dans les graines et les racines. — *Rev. gen. bot.* 61: 637. 1954.
11. — & Meylan, S.: Polarité électrique, auxines et physiologie des racines du *Lens culinaris*. — *Bul. soc. bot. suisse* 63: 430. 1953.
12. — & Went, F. W.: Control of growth of *Lens culinaris* by temperature and light. — *Amer. Jour. Bot.* (in preparation).
13. Siegel, S. M. & Galston, A. W.: Peroxide genesis in plant tissues and its relation to indoleacetic acid destruction. — *Arch. Biochem. & Biophys.* 54: 102. 1955.
14. Tang, Y. W. & Bonner, J.: The enzymatic inactivation of indoleacetic acid I. Some characteristics of the enzyme contained in pea seedlings. — *Arch. Biochem. & Biophys.* 13: 11. 1947.
15. Andreae, W. A. & Andreae, S. R.: Studies on indoleacetic acid metabolism I. — *Can. J. Bot.* 31: 426. 1953.

A Physiological Comparison of Gibberellic Acid with Some Auxins

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Introduction

Gibberellic acid, if applied to plants through their roots or foliage, commonly induces a marked increase in stem internode length and, in the case of cereals, in leaf length (Brian, Elson, Hemming and Radley, 1954; Brian and Hemming, 1955). This is attributable to increased cell growth rather than to increased cell division. In this respect gibberellic acid invites comparison with the auxins. We describe below a series of physiological comparisons of gibberellic acid with indolylacetic acid and other auxins. Comparisons of gibberellin A (a substance very similar in its properties to gibberellic acid) with auxins have been made by Sumiki (1952), Kato (1953) and Hayashi and Murakami (1953 a, 1953 b, 1954).

Materials and Methods

Wheat coleoptile section extension. The technique of Luckwill (1952) was used. In the experiment quoted, 8 replicates of 10 sections were used for each treatment. The initial length of section was 2.25 mm. For statistical analysis the measurements of the ten sections in each replicate were added.

Pea stem section test. The technique of Miller (1954) was used. Sections 5.4 mm. long were cut from the third internode of Meteor pea seedlings grown in darkness. For each treatment 4 replicates of 5 sections were used, and for statistical treatment

the data from the five sections in each replicate were added. Gibberellic acid, indolylacetic acid and cobaltous chloride (see Table 2) were made up as aqueous solutions all containing 2 per cent sucrose. The tests were carried out at 25° C. in the dark.

Water uptake by potato tuber tissue. The method of Hackett and Thimann (1952) was used, the experimental material being circular discs, 10 mm. in diameter and 1 mm. in thickness, of 'King Edward' potato tuber tissue. In the experiments described each treatment was replicated three times, each replicate consisting of 10 discs, each group of 10 being weighed together.

Cress root growth. The method was essentially that of Audus (1951), but the root length of all seedlings was measured, not merely that of the largest† in each replicate. Each treatment was replicated 10 times, each replicate consisting of 20 seedlings. For statistical analysis the root lengths of all seedlings in a replicate were added.

Inhibition of lateral buds. 'Meteor' pea seedlings, 14 days old, were decapitated immediately above the fifth node. Lanoline pastes containing indolylacetic acid, gibberellic acid or 2:4-dichlorophenoxyacetic acid, of the concentrations stated in the text below, were applied to the cut surface. After 8 days the lateral buds or shoots were cut off and their length measured. Similar measurements were made of buds on untreated plants and on plants decapitated and with lanoline alone applied to the cut surface. Each treatment was applied to 24 plants, in six pots containing groups of 4 plants. For statistical analysis the data for the four plants in each pot were added so that in effect each treatment was replicated six times; the replicates were arranged in randomised blocks on the greenhouse bench.

Dormancy of potato tubers. Methods are described in the text.

Petiole abscission. Abscission was studied using the *in vitro* *Coleus* explant technique of Addicott, Lynch, Livingston and Hunter (1949). Their technique was modified in two ways: — (a) instead of using isolated nodes with a pair of attached petioles, these were split longitudinally so that each explant carried only one petiole; (b) the substances to be tested were applied to the distal end of the petiole in 0.001 ml. ethyl alcohol.

Root initiation. Shoots of Meteor peas, 3 weeks old, were cut off at the base and the lower leaves removed. These were stood in a 1 in. depth of the auxin or gibberellic acid solution for 24 hr., then removed and placed in a similar depth of water. Observations on rooting were made at intervals for 2 weeks.

Abbreviations. The following abbreviations are used: — GA, gibberellic acid; 2:4-D, 2:4-dichlorophenoxyacetic acid; IAA, 3-indolylacetic acid; NAA, 1-naphthylacetic acid.

Results

Extension of wheat coleoptile sections

IAA and GA were compared in concentrations from 0.01 to 100.0 µg./ml. (Table 1). Two points are illustrated which we have confirmed in numerous experiments, *viz.* (a) at 0.01 µg./ml. GA induces a greater extension than IAA; (b) further increase in the concentration of GA does not cause increased

Table 1. *Mean final length (mm.) of 10 wheat coleoptile sections (initial length 22.5 mm.) after 24 hr. in aqueous solutions of gibberellic acid (GA) or indolylacetic acid (IAA).*

Substance	Concentration (µg./ml.)				
	0.01	0.1	1.0	10.0	100.0
GA	36.6	36.8	37.1	36.4	35.6
IAA	34.9	37.3	39.6	40.1	39.1

Water controls: 35.0, 34.5

Significant difference ($S.E. \times t \times \sqrt{2}$) = 1.0 ($P=5\%$), 1.4 ($P=1\%$).

extension, whereas the effect of IAA increases as the concentration is increased up to 10 µg./ml.

Hayashi and Murakami (1953 b) obtained similar results with gibberellin A in an oat coleoptile section test, but were unable to detect any significant response in wheat coleoptile sections; however, their wheat coleoptile test was much less sensitive than their oat coleoptile test.

Extension of etiolated pea stem sections

IAA and GA were compared in concentrations of 0.1, 1 and 10 µg./ml., with or without added cobaltous chloride (Table 2). As in the wheat coleoptile test GA was more effective than IAA in low doses (0.1 µg./ml.); also here

Table 2. *Mean final length (mm.) of 5 etiolated pea stem sections (initial length 27.0 mm.) after 24 hr. in solutions of gibberellic acid (GA) or indolylacetic acid (IAA), with or without additional cobalt chloride (10^{-4} M).*

Treatment	Without CoCl_2	With CoCl_2	Mean
Water	40.3	41.4	40.9
»	42.5	42.1	42.3
GA, 0.1 µg./ml.	47.6	48.8	48.1
1.0 µg./ml.	46.1	44.4	45.3
» 10 µg./ml.	47.5	49.5	48.5
IAA, 0.1 µg./ml.	43.8	44.6	44.1
» 1.0 µg./ml.	42.6	44.9	43.8
» 10 µg./ml.	45.4	49.1	47.3
Mean	44.4	45.6	

Significant differences ($S.E. \times t \times \sqrt{2}$)

For comparison of individual means 3.4 ($P=1\%$), 2.6 ($P=5\%$)
 » » means in side margin 2.4 ($P=1\%$), 1.8 ($P=5\%$)
 » » » lower margin 1.2 ($P=1\%$), 0.9 ($P=5\%$)

Table 3. *Percentage increase in fresh weight of potato tuber discs in aqueous solutions of 1-naphthylacetic acid (NAA), indolylacetic acid (IAA) or gibberellic acid (GA) at 25° C. (Significant differences are based on analysis of variance of data after angular transformation.)*

Experiment	Treatment	Days		
		3	5	7
I	Water only	10.2	15.2	19.0
	NAA, 10 µg./ml.	13.9	30.5 ²	46.3 ²
	NAA, 1 µg./ml.	11.2	24.7 ²	41.6 ²
	IAA, 10 µg./ml.	13.0	20.4 ¹	27.0 ¹
	IAA, 1 µg./ml.	12.1	15.2	20.9
	GA, 10 µg./ml.	10.2	13.6	16.1
	GA, 1 µg./ml.	10.7	15.2	18.5
II	Water only	12.0	16.9	18.7
	GA, 100 µg./ml.	11.9	15.1	17.1
	GA, 10 µg./ml.	12.8	16.5	18.6
	GA, 1 µg./ml.	11.6	15.9	19.0
	GA, 0.1 µg./ml.	11.7	16.6	17.8
	GA, 0.01 µg./ml.	10.8	15.2	17.1
	NAA, 10 µg./ml.	20.4 ²	47.0 ²	63.8 ²

Significantly different from water only, ¹ $P < 5\%$, ² $P < 1\%$.

again further increase in GA concentration did not lead to an increased response. However, in this test the response induced by 10 µg./ml. IAA, while greater than that induced by 0.1 µg./ml. IAA, was less than that induced by 0.1 µg./ml. GA. This is in contrast to the results of Hayashi and Murakami (1953 a) with gibberellin A, which was found to be less active than IAA. Cobaltous chloride slightly increased extension in this experiment, as demonstrated earlier by Miller (1954).

Water uptake by potato tuber tissue

NAA increased water uptake (Table 3), as measured by increase in fresh weight, very considerably; IAA had a much smaller but just significant effect. This is in agreement with the results of Hackett and Thimann (1952). GA had no detectable effect.

Root growth

The effect of GA and IAA on root growth of cress seedlings was studied in a factorial experiment in which each substance was tested at four levels in all possible combinations; the results are summarised in Table 4. The root lengths recorded varied widely from treatment to treatment and, since the range of replicate observations in any one treatment tended to be proportional to the mean, it was judged necessary to transform the primary data before statistical analysis; a logarithmic transformation was used (Table 5).

Table 4. *Mean length (mm.) of roots of cress seedlings growing in aqueous solutions containing gibberellic acid (GA) and indolylacetic acid (IAA).*

IAA concentration µg./ml.	GA concentration (µg./ml.)				Mean
	0	0.1	1.0	10.0	
0	28.9	26.9	29.3	24.7	27.4
0.1	15.2	16.4	16.0	12.7	15.1
1.0	5.5	6.1	6.0	7.8	6.3
10.0	3.8	3.6	3.8	3.6	3.7
Mean	13.4	13.2	13.8	12.2	

The well-known inhibiting effect of IAA on root growth was clearly demonstrated, the low concentration of 0.1 µg./ml. reducing mean root length to approximately half that of the untreated seedlings. GA had no significant effect on root growth at any of the concentrations tested. In other experiments we have found that concentrations as high as 100 µg./ml. have little inhibitory effect on root growth. There is evidence from the analysis of an interaction between GA and IAA. Though statistically significant the effect is small.

Table 5. *Cress root growth test (see Table 4): statistical analysis of data after logarithmic transformation.*

IAA concentration µg./ml.	GA concentration (µg./ml.)				Mean
	0	0.1	1.0	10.0	
0	2.76	2.72	2.77	2.69	2.74
0.1	2.48	2.51	2.50	2.40	2.47
1.0	2.03	2.08	2.07	2.19	2.09
10.0	1.87	1.86	1.87	1.85	1.86
Mean	2.29	2.29	2.30	2.28	

Significant difference ($S.E. \times t \times \sqrt{2}$) for comparison of individual means 0.08 ($P=5\%$),
0.10 ($P=1\%$).

» » » for comparison of marginal means 0.04 ($P=5\%$),
0.05 ($P=1\%$).

Analysis of variance

Effects	Degrees of freedom	Sum of squares	Mean square
GA main effect ...	3	0.01	0.003
IAA main effect ...	3	18.03	6.010 ²
Interaction	9	0.24	0.027 ²
Residual error ...	144	1.10	0.008
Total	159	19.38	

² Variance ratio significant at 1 % level.

Table 6. *Number of non-inhibited lateral buds on 24 Meteor pea seedlings, 8 days after decapitation and application of lanoline pastes containing indolylacetic acid (IAA), gibberellic acid (GA) or 2:4-dichlorophenoxyacetic acid (24D) to the cut shoot apex.*

Treatment	Node					Total for all nodes
	I	II	III	IV	V	
Lanoline only	13	24	24	24	24	109
IAA, 0.1 mg./g.	7	24	22	21	20	93
» 1.0 mg./g.	1	22	22	17	14	76
» 10 mg./g.	1	19	6	0	0	26
GA, 0.1 mg./g.	10	24	22	21	21	98
» 1.0 mg./g.	17	24	19	18	22	100
» 10 mg./g.	17	24	19	21	23	104
24D, 0.1 mg./g.	16	24	22	21	21	103
» 1.0 mg./g.	6	22	11	2	8	49
» 10 mg./g.	0	6	0	0	2	8
Not decapitated and untreated	0	0	0	0	0	0

Sumiki (1952) found that in the range 0.02—10.0 µg./ml. gibberellin A had no marked effect on the development and growth of oat-roots: his data were based on a limited number of observations and no statistical treatment was attempted.

Inhibition of lateral buds

The results are presented in two ways: — (a) the lateral buds were graded as inhibited or non-inhibited, any buds greater than 3 mm. in length being arbitrarily taken to be non-dormant; the number of non-inhibited buds at each node in the 24 experimental plants included in each treatment is given in Table 6: (b) the length of the lateral buds or shoots was measured; the mean length of these buds or shoots at each node is presented in Table 7, with an indication of those significantly differing in length from those of decapitated plants treated with lanoline only.

All buds in the intact plants remained inhibited. Decapitation released from inhibition all buds at nodes II to V, but not all those at node I. (It has been noticeable in many experiments that the bud at node I is much more strongly inhibited than any others in 'Meteor' pea seedlings, whereas that at node II is the least inhibited). The bud at node II produced a much longer shoot than any other bud.

Application of a lanolin paste containing 0.1 mg./g. IAA to the cut shoot apex had little effect. The paste containing 1 mg./g. had a marked inhibitory effect on all lateral buds, and the paste containing 10 mg./g. retarded bud development to virtually the same level as on plants with an intact apical bud. This essentially reproduces the results of Skoog and Thimann (1934). Rather unexpectedly the effect of 2:4-D differs from that of indolylacetic

Table 7. Mean length (mm.) of lateral buds or shoots on Meteor pea seedlings, 8 days after decapitation and application of lanoline pastes containing indolylacetic acid (IAA), gibberellic acid (GA) or 2:4-dichlorophenoxyacetic acid (24D) to cut shoot apex. (Significant differences are based on analysis of variance of data after logarithmic transformation).

Treatment	Node					Total for all nodes
	I	II	III	IV	V	
Lanoline only	4.9	38.0	15.8	13.4	12.8	84.9
IAA, 0.1 mg./g.	7.5	40.6	18.4	8.6	9.0	84.1
» 1.0 mg./g.	1.5 ²	14.3 ²	11.3	5.6 ²	5.4 ²	38.1 ²
» 10 mg. g.	1.5 ²	4.2 ²	3.0 ²	1.6 ²	0.3 ²	10.6 ²
GA, 0.1 mg./g.	8.5	47.6	17.7	12.3	18.4	104.5
» 1.0 mg./g.	11.0 ¹	96.1 ²	28.8	8.2 ²	19.8	163.9 ²
» 10 mg. g.	10.5 ¹	89.6 ²	17.9	9.1	18.5	145.6 ²
24D, 0.1 mg. g.	8.9	69.6 ¹	19.1	6.7 ²	17.6	122.2 ¹
» 1.0 mg. g.	3.1 ¹	39.9	11.1 ¹	1.8 ²	3.1 ²	59.0 ²
» 10 mg. g.	1.4 ²	2.7 ²	1.4 ²	0.7 ²	0.9 ²	7.1 ²
Not decapitated and untreated	1.1 ²	2.0 ²	1.1 ²	0.3 ²	0.3 ²	5.3 ²

¹ Significantly different ($P < 5\%$) from lanoline controls.

² » » » ($P < 1\%$) » » »

acid in some respects. The paste containing 0.1 mg./g. very markedly stimulated the development of lateral shoots at node II, while having little influence on the buds at other nodes: we have confirmed this result in other experiments. The paste containing 1 mg./g. 2:4-D inhibited buds at all nodes except node II, which was unaffected. The paste containing 10 mg./g. 2:4-D inhibited buds at all nodes, behaving similarly to the comparable indolylacetic acid paste. Both IAA and 2:4-D, especially at the higher rates of application, caused marked stem thickening, especially near the point of application.

GA produced a rather different response. The paste containing 0.1 mg./g. had little effect but the two more concentrated pastes caused a noticeable increase in the length of lateral shoots especially at node II and to a somewhat smaller extent at node I. While this response was quite distinct from that elicited by IAA or the higher concentrations of 2:4-D, it was rather similar to the response to the lowest dose of 2:4-D. Gibberellic acid caused no thickening of the stem.

Kato (1953) found that gibberellin A stimulated and IAA inhibited the development of cotyledonary buds in decapitated pea (etiolated) and dwarf bean seedlings.

Table 8. *Effect of gibberellic acid soak treatment of dormant potato tubers on sprouting* (GA₁₀=10 µg./ml. solution of gibberellic acid, GA₁₀₀=100 µg./ml.).

Experimental details	Water 2 hr.	Water 24 hr.	GA ₁₀ 2 hr.	GA ₁₀₀ 2 hr.	GA ₁₀ 24 hr.	GA ₁₀₀ 24 hr.
1. <i>Majestic</i> (50 tubers ¹)						
No. sprouted (64 days)	27	—	45	45	—	—
Mean no. of shoots per tuber (64 days)	2.0	—	5.4	4.6	—	—
2. <i>Epicure</i> (40 tubers ¹)						
No. sprouted (11 days)	—	22	28	30	29	40
No. sprouted (28 days)	—	40	40	40	40	40
Mean no. shoots per tuber (28 days) ...	—	5.9	7.3	9.1	9.1	10.0
Fresh weight of shoots, g. (28 days) ...	—	1.4	6.4	9.3	7.7	11.4
3. <i>Majestic</i> (28 tubers ¹)						
No. sprouted (10 days)	—	2	8	11	14	16
No. sprouted (31 days)	—	14	23	25	27	27

¹ no. tubers per treatment.

Breaking of dormancy of potato tubers

We have carried out only three small experiments (Table 8) but the results were quite conclusive.

(1) 'Majestic' potato tubers were soaked in GA (100 µg./ml. and 10 µg./ml.) for 2 hr., potatoes soaked in water for a similar period being used as controls. The treatment was carried out in October, 1954, soon after the tubers had been lifted so that they were consequently in a highly dormant condition. After soaking the tubers were kept in a heated glasshouse. The tubers treated with GA began to sprout within 2 weeks while the control tubers were still completely dormant. After 64 days almost all the tubers treated with GA had sprouted vigorously, whereas only half the controls had sprouted. The shoots on GA treated tubers were more numerous, less confined to the apex of the tuber, and were more richly branched than those on control tubers. Their appearance strongly suggested a loss of apical dominance.

(2) 'Epicure' tubers were treated in November, 1954; in this case 2 hr. and 24 hr. soaks were tested. These tubers, being of an early variety, were noticeably less dormant than the 'Majestic' but GA had a noticeable dormancy-breaking effect. Branching of the shoots produced was again noticed.

(3) 'Majestic' tubers were treated in December, 1954, and stimulation of sprouting by GA was again very noticeable (Table 8).

This dormancy-breaking effect is to be contrasted with the characteristic dormancy inducing effect of IAA (Guthrie, 1939).

Table 9. *Effect of application of indolylacetic acid (IAA) and gibberellic acid (GA) on abscission of Coleus petioles: no of petioles shed, from total of 10 in each case.*

Treatment	Days after treatment				
	1	2	3	5	6
IAA, 100 µg.	0	0	1	2	5
» 10 µg.	0	0	0	0	3
» 1 µg.	0	0	0	1	4
» 0.1 µg.	0	2	6	8	10
GA, 100 µg.	10	10	10	10	10
» 10 µg.	10	10	10	10	10
» 1 µg.	10	10	10	10	10
» 0.1 µg.	10	10	10	10	10
Untreated	10	10	10	10	10
»	10	10	10	10	10

Coleus petiole abscission

The results are presented in Table 9. Even the lowest dose of IAA, 0.1 µg., delayed abscission considerably. GA, on the other hand, did not delay abscission at all in the dose range 0.1—100.0 µg.

Cell division

In experiments where lanoline pastes containing 2:4-D or IAA were applied to decapitated pea seedlings (Tables 6 and 7) or to tomato or broad bean (*Vicia*) seedlings stem thickening invariably developed; 2:4-D produced more severe and less localized swellings than IAA. Swellings of this type, caused by excessive cell proliferation in cambium or cortex, are a common feature of auxin action, but we have never observed any such developments after application of GA, even in very large doses.

Sumiki (1952) and Kato (1953) showed that gibberellin A did not induce callus formation on cut stem surfaces of broad bean, sunflower or tomato, whereas IAA and NAA had a pronounced effect.

Root initiation on stem cuttings

The results of an experiment on rooting of stem cuttings of Meteor pea are summarised in Table 10. IAA and NAA both induced rooting; GA did not, and in fact reduced rooting. It was noteworthy that the 24 hr. treatment with GA led to considerably elongation of the main axis of the stem cuttings.

Table 10. *Effect on root initiation of Meteor pea stem cuttings of 24 hr. treatments with indolylacetic acid (IAA), 1 naphthylacetic acid (NAA) and gibberellic acid (GA). (Observations 14 days after treatment).*

Treatment	No. of cuttings rooted (possible maximum: 40)	Mean no. of roots per rooted cutting
Untreated	10	3.1
IAA, 10 μ g./ml.	29	3.5
» 1 μ g./ml.	12	2.1
NAA, 10 μ g./ml.	32	7.1
» 1 μ g./ml.	21	4.1
GA, 10 μ g./ml.	0	0
» 1 μ g./ml.	1	1.0
» 0.1 μ g./ml.	1	1.0

Discussion

One of the most striking features of the physiological activity of IAA is the multiplicity of responses which it induces in plant tissues. These responses include increased cell-extension in shoot tissues, increased cambial activity, stimulation of root initiation, inhibition of the growth of roots and lateral buds and prevention of petiole abscission. This multiplicity of response has led Thimann (1948) to postulate that IAA takes part in some fundamental 'master reaction' in the cell. The majority of synthetic auxins elicit a similar multiple response.

Is GA an auxin? Thimann (1948), Tukey, Went, Muir and van Overbeek (1954) and Larsen (1954) agree that an auxin should be defined primarily by its capacity to induce elongation in shoot cells. In so far as GA induces elongation of shoot cells of intact plants and of wheat coleoptile or pea stem sections, it must be considered to be an auxin or an auxin precursor. We have at present no reason to suppose that it is a precursor rather than an actual auxin so in the succeeding argument that possibility is not considered, though it may eventually be necessary to do so. The failure of GA to stimulate water uptake by potato tuber tissue needs to be considered here. This test works particularly well with NAA, which induces greatly increased water uptake. In our experience, confirming that of Hackett and Thimann (1952), IAA causes much smaller increases, which in our experiments only just reach statistical significance. If GA induced a smaller response than IAA, as it does in the wheat coleoptile section test, we could not expect to detect it by the potato disc technique without a much greater amount of experimental replication. Consequently we do not consider that the negative result obtained in this test significantly detracts from our conclusion that GA is an auxin.

It is convenient to summarise here the similarities and the differences between GA and IAA. Both stimulate shoot cell extension; here their similarity apparently ends. Unlike IAA, GA does not inhibit root growth or the development of lateral buds; it does not prevent petiole abscission, neither does it stimulate cambial activity or initiation of roots on stem cuttings. Thus, whereas auxins in general are characterised by a multiplicity of physiological responses, GA is characterised by specificity, the only growth response yet detected being increased cell extension in shoot tissues. This appreciation of the activity of GA is supported by the results obtained with the related substance gibberellin A by Japanese workers. We are naturally led by these differences in physiological activity to enquire whether the modes of action of GA and IAA are related. This does not conflict with our earlier conclusion that GA is an auxin; this is no more than a conclusion that both produce a similar end effect on shoot cell growth and it does not necessarily follow that they intervene in the growth process at the same stage.

On further examination some of the differences between GA and IAA appear to be differences of degree rather than of kind. At first sight, the failure of GA to inhibit development of lateral shoots in decapitated pea seedlings might be considered to be an important distinction from the auxins. In this respect GA certainly differs from IAA and the higher doses of 2 : 4-D, but we have shown that in low doses the auxin 2 : 4-D behaves similarly to GA in so far as it increases the growth of the lower lateral shoots. The distinction from auxins in this experiment is therefore not absolute. Similarly, while the potato tuber dormancy-breaking effect of GA at first sight appears contrasted with the well-known sprout-inhibiting properties of such auxins as IAA and NAA, Guthrie (1939) has shown that low concentrations of IAA may have a slight dormancy breaking effect on potato tubers. Thus in these effects on buds, whether of tubers or of decapitated shoots, GA and the auxins could be interpreted as behaving similarly, GA having a stimulating effect over a wider range of concentrations than such auxins as IAA or 2 : 4-D. Indeed, a feature of the biological activity of GA is its lack of inhibitory effect in high doses. We have shown (Brian *et al.*, 1955) that in experiments with intact Meteor pea plants an appreciable stimulation of growth is produced by application of GA in a dose of 0.01 $\mu\text{g.}$ per plant and that a dose of about 1 $\mu\text{g.}$ produced a maximal response. We have since found (unpublished results) that a dose of 1000 $\mu\text{g.}$ per plant produced an effect of the same order as that produced by 1 $\mu\text{g.}$ In other words, a dose 10^5 times greater than that required to produce an obvious growth response was still growth-promoting.

A more weighty distinction from the auxins is in the effect on root growth. It is characteristic of most auxins that, at concentrations which increase

growth of coleoptile sections, root growth is strongly inhibited. GA had no significant effect in the concentration range 0.1—10 $\mu\text{g./ml.}$ Root growth is a complex process involving cell division and cell extension and the inhibitory action of auxins involves inhibition of both these processes. Effects of GA on cell division are discussed below; the failure of GA to inhibit root cell extension can possibly be explained by the generally non-toxic nature of high doses as in the case of lateral buds.

Thus in general terms, in so far as effects on cell-extension are concerned, there seems no reason to suppose that the physiological activity of GA and more well known auxins are different in nature. However, in its failure to prevent petiole abscission, to stimulate cell-division in cambial or cortical tissues or the stem, or to stimulate rooting of shoot cuttings, GA differs sharply from IAA and most auxins. It is noteworthy that stimulation or inhibition of cell division is involved in these physiological responses. It is tempting to conclude that GA is different from the auxins in mode of action, acting either by affecting the growth process at a stage subsequent to Thimann's postulated master reaction, so that the generalised response involving both the cell division and cell extension phenomena characteristic of IAA is not produced, or by affecting cell-extension by some entirely different mechanism. Alternatively it is possible that IAA and auxins of analogous chemical structure affect cell-division and cell-extension by entirely different mechanisms, in only one of which can the auxin be replaced by GA. However, there is as yet no positive evidence in favour of any of these speculative schemes. The only reason at present for considering the possibility that GA has a distinct mode of action lies in its greater specificity, in other words on the purely negative evidence that it does not do some of the things which IAA does. In this connection it is apposite to recall that indolylacetonitrile, which occurs naturally in some species of plants, shows a much greater specificity in the growth responses it evokes than does IAA, but it is nevertheless likely that the same mode of action is involved. Indolylacetonitrile is active in inducing oat coleoptile section extension, in the initiation of cambial activity in *Salix*, and in inhibition of growth of oats and cress roots; it is inactive in promoting growth of pea epicotyl sections, in inhibition of lateral buds of *Phaseolus*, in root initiation in *Phaseolus*, and in inhibition of petiole abscission in apple (Bentley and Bickle, 1952; Bentley and Housley, 1952, 1953; Thimann, 1953). All these differences from IAA, whether involving greater or less activity than IAA, can be plausibly accounted for by two factors — greater rapidity of entry of the neutral indolylacetonitrile molecule into the cell, and the need for metabolic hydrolysis to IAA before a response is obtained, the necessary hydrolytic enzyme system being present only in certain plant species. While the specificity of action of indolylaceto-

nitrile is less marked than that of GA, and while the specificity of GA cannot be accounted for in the same way, this example shows that specificity is not necessarily indicative of a distinct mode of action for GA; further positive evidence is required before such a conclusion would be really justified.

Summary

- (1) Gibberellic acid (GA) increased extension growth of wheat coleoptile sections. It elicited a maximum response in concentrations of 0.01 $\mu\text{g./ml.}$ Further increase in concentration to 100 $\mu\text{g./ml.}$ did not significantly increase or decrease response from this level. At 0.01 $\mu\text{g./ml.}$ GA was more active than indolylacetic acid (IAA) at the same concentration. Optimum concentrations of IAA (*ca.* 10 $\mu\text{g./ml.}$) induced a much greater response than GA. GA was more effective than IAA in inducing extension of pea stem sections, but increase in concentration of GA above 0.1 $\mu\text{g./ml.}$ did not increase or decrease response. GA did not increase water uptake by potato tuber tissue.
- (2) When applied to decapitated pea seedlings GA at all concentrations tested stimulated the growth of lateral shoots from the lower leaf axils of the main stem. In this it differed from IAA which inhibited lateral shoot development at all nodes. 2:4-Dichlorophenoxyacetic acid in low doses behaved rather similarly to GA, but in higher doses it inhibited lateral buds as effectively or more effectively than IAA.
- (3) GA did not inhibit the growth of cress roots; it did not delay abscission of *Coleus* petioles; it did not stimulate cell division in pea, broad bean (*Vicia*) or tomato stems; not only did it fail to stimulate root initiation in pea stem cuttings but it appeared to inhibit root initiation. In all of these respects it differed from IAA.
- (4) GA must be considered to be an auxin in the sense of the recent definition of Tukey *et al.* (1954). It fails to elicit many of the physiological responses characteristic of IAA, particularly those in which cell division is involved. In the absence of more direct evidence, it is considered premature to conclude that its mode of action in promoting shoot cell growth is distinct from that of IAA and other auxins.

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References

- Addicott, F. T., Lynch, R. S., Livingston, G. A. & Hunter, J. K.: A method for the study of foliar abscission *in vitro*. — *Plant Physiol.* 24: 537. 1949.

- Audus, L. J.: The biological detoxication of hormone herbicides in soil. — *Plant and Soil*, 3: 170. 1951.
- Bentley, J. A. & Bickle, A. S.: Studies on plant growth hormones. I. Biological activities of 3-indolylacetaldehyde and 3-indolylacetonitrile. — *J. Exp. Bot.* 3: 393. 1952.
- & Housley, S.: Studies on plant growth hormones. II. Further biological properties of 3-indolylacetonitrile. — *J. Exp. Bot.* 3: 406. 1952.
- — Growth of Avena coleoptile sections in solutions of 3-indolylacetic acid and 3-indolylacetonitrile. — *Physiol. Plant.* 6: 480. 1953.
- Brian, P. W., Elson, G. W., Hemming, H. G. & Radley, M.: The plant-growth-promoting properties of gibberellic acid, a metabolic product of the fungus *Gibberella fujikuroi*. — *J. Sci. Food Agric.* 5: 602. 1954.
- & Hemming, H. G.: The effect of gibberellic acid on shoot growth of pea seedlings. — *Physiol. Plant.* 8: 669. 1955.
- Guthrie, J. D.: Control of bud growth and initiation of roots at the cut surface of potato tubers with growth regulating substances. — *Contr. Boyce Thompson Inst.* 11: 29. 1939.
- Hackett, D. P. & Thimann, K. V.: The nature of the auxin-induced water uptake by potato tissue. — *Amer. J. Bot.* 39: 553. 1952.
- Hayashi, T. & Murakami, Y.: The biochemistry of the bakanae fungus. Part 29. The physiological action of gibberellin (5). The effect of gibberellin on the straight growth of etiolated pea epicotyl sections. — *J. agric. chem. Soc. Japan*, 27: 675. 1953 a. — (Title translated from Japanese.)
- — The biochemistry of the bakanae fungus. Part 30. The physiological action of gibberellin (6). The effect of gibberellin on the straight growth of isolated sections of cereal coleoptiles. — *J. agric. chem. Soc. Japan*, 27: 797. 1953 b. — (Title translated from Japanese.)
- — The biochemistry of the bakanae fungus. Part 32. The physiological action of gibberellin. (7). Response of different parts of cereal leaves to gibberellin. — *J. agric. chem. Soc. Japan*, 28: 543. 1954. — (Title translated from Japanese.)
- Kato, J.: Studies on the physiological effect of gibberellin. I. On the differential activity between gibberellin and auxin. — *Mem. Coll. Sci. Kyoto, Ser. B.* 20: 189. 1953.
- Larsen, P.: Nomenclature of chemical plant regulators. — a criticism. — *Amer. J. Bot.* 29: 400. 1954.
- Luckwill, L. C.: Application of paper chromatography to the separation and identification of auxins and growth-inhibitors. — *Nature, Lond.* 109: 375. 1952.
- Miller, C. O.: The influence of cobalt and sugars upon the elongation of etiolated pea stem segments. — *Plant Physiol.* 29: 79. 1954.
- Skoog, F. & Thimann, K. V.: Further experiments on the inhibition of lateral buds by growth hormone. — *Proc. Nat. Acad. Sci. Washington*, 20: 480. 1934.
- Sumiki, Y.: The biochemistry of the bakanae fungus. Part 25. The physiological action of gibberellin (3). — *J. agric. chem. Soc. Japan*, 26: 393. 1952. — (Title translated from Japanese.)
- Thimann, K. V.: Plant Growth Hormones. In *The Hormones*, by G. Pincus and K. V. Thimann, Vol. 2. — Academic Press, New York. 1948.
- Hydrolysis of indoleacetonitrile in plants. — *Arch. Biochem. Biophys.* 44: 242. 1953.
- Tukey, H. B., Went, F. W., Muir, R. M. & van Overbeek, J.: Nomenclature of chemical plant regulators. — *Amer. J. Bot.* 29: 307. 1954.

The Effect of Various Cycles of Light and Darkness on the Growth of Tomato and Cocklebur Plants

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Introduction

Garner and Allard (2) showed in 1931 that alternating periods of light and darkness of equal length and from 5 seconds to 1 hour in duration have effects on the growth of plants different from those of a 12-hour »control«, even though in a 24-hour period the plants under the various treatments all receive the same total amount of light. These growth variations were found with all the eleven species of short-day, long-day, and indifferent plants studied, with differences also noted in the time required for flowering under the various light-dark cycles. The studies were begun with young seedlings and lasted from 20 to 98 days. Variations in heights of the plants at the end of the test periods were reflected in variations in fresh and dry weights of tops and roots. These effects were further studied by Highkin and Hanson (2) with 6, 12, and 24-hour alternations of light and darkness, and these workers found a striking inhibition of growth of the tomato grown from seed with the 6-hour and 24-hour cycles over a period of about 6 weeks. Peas showed some inhibition, while sunflowers were not significantly affected.

The present work was taken up to determine the effects of various cycles on the growth of tomato and cocklebur plants over a shorter period of time and with various light intensities. In this paper a cycle of 2 hours of light

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and 2 hours of darkness, for example, will be called a »2-hour cycle», the alternating light and dark periods in each treatment always being of the same length.

Materials and Methods

Seeds of tomato (variety Extra Early) and burs of cocklebur (*Xanthium pennsylvanicum*) were sown in vermiculite and allowed to germinate. About a week later the seedlings were transplanted into cups in vermiculite and watered regularly with nutrient solution. The tomato seedlings were grown after transplanting for about two weeks in a greenhouse at a day temperature (8 hours) of 23° C. and a night temperature (16 hours) of 17° C., while the cocklebur seedlings were grown at a day temperature of 26° C. and a night temperature of 20° C. for a similar length of time. The cockleburs were given supplemental light every day from 4:00 to 12:00 pm to keep them from flowering.

The »light boxes» used to give the alternating periods of light and darkness to the test plants were arranged in a row of five. The tops of these boxes were opened and closed by electric motors which were controlled by time clocks. The motors pulled the sliding covers off the boxes or replaced them at the appropriate times. Each box held 3 racks of 9 test plants each, and these racks could be placed at various distances from the tops of the boxes to vary the light intensity received by the 3 groups of plants. The light source directly above the row of boxes consisted of warm white fluorescent tubes interspersed with incandescent bulbs. This artificial light source gave an intensity of about 1500 foot-candles (fc) near the tops of the boxes. The temperature of the room in which the ventilated boxes were located was kept at 17° C.

The experimental plants were selected at random into groups of 9 from a lot of plants uniform in size after the tallest and shortest ones had been discarded from the initial population. The plants were grown under the experimental conditions for 2 to 3 weeks. At harvesting the total heights and fresh and dry weights of roots and tops separately were determined, with an average in each case being taken of the 9 plants in each treatment.

Young cocklebur and tomato plants were tested with various light intensities in combinations of 5-minute, 15-minute, 1-hour, 2-hour, 4-hour, 6-hour, 9.6-hour, 12-hour, and 24-hour cycles. The flowering cockleburs used in one experiment were induced to flower by exposure to the natural short daylength of late October for about 2 weeks in the greenhouse. Flower buds were detectable when the plants were put into the light boxes. Chlorophyll was extracted from leaf stamps with methanol, and the relative concentrations of the extracts were determined with a colorimeter.

Results

Figures 1, 2, and 3 show the effects of 2-hour, 4-hour, 6-hour, 9.6-hour, and 12-hour cycles on growth of tomato plants as measured by increase in height, fresh weight, and dry weight of the tops over a period of 17 days in

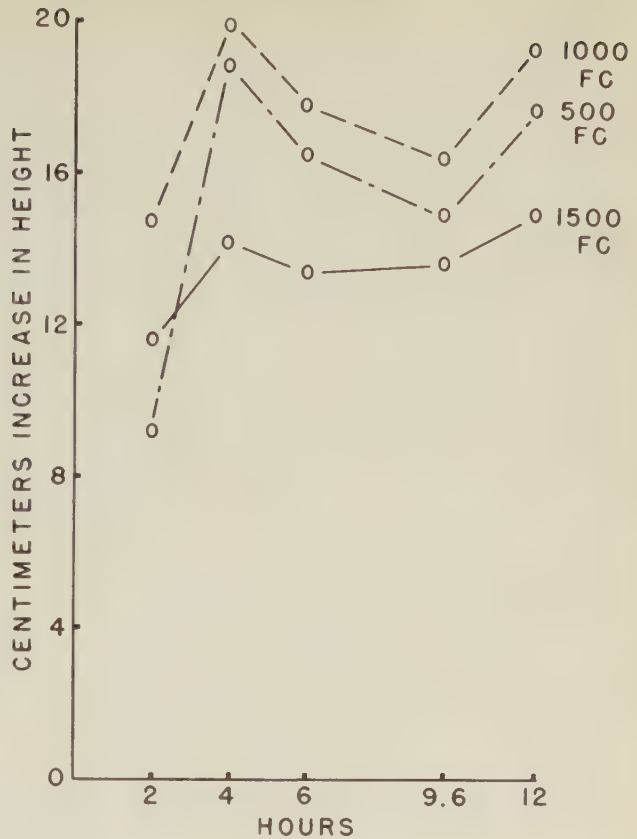


Figure 1. *Average increase in height in 17 days of tomato plants grown under various cycles of light and darkness at three light intensities. Figures on the abscissa indicate the length of each light period or its alternating dark period. Each point on the graphs represents an average of 9 plants.*

the light boxes at light intensities of 500, 1000, and 1500 fc. The graphs of the fresh weight and dry weight increases show essentially the same pattern under the 5 cycles, and the graphs for each of the three light intensities employed are also quite similar. However, the increase in height of the plants (Figure 1) was considerably greater in the 12-hour cycle than in the 9.6, whereas these two cycles show about the same production of fresh and dry weight (Figures 2 and 3). With the other series of cycles used in this study the three graphs (increase in height, fresh weight, and dry weight) were more nearly alike, and only the data for dry weight increase of the tops will be given as a measure of growth. The most growth in height was usually shown by the plants grown at 1000 fc and the least at the highest intensity of 1500 fc, while increases in fresh and dry weight were greatest at the highest intensity and smallest at the lowest intensity.

Figure 3 shows that of the 5 cycles employed in this particular series with the tomato, growth as measured by dry weight increase was greatest in the

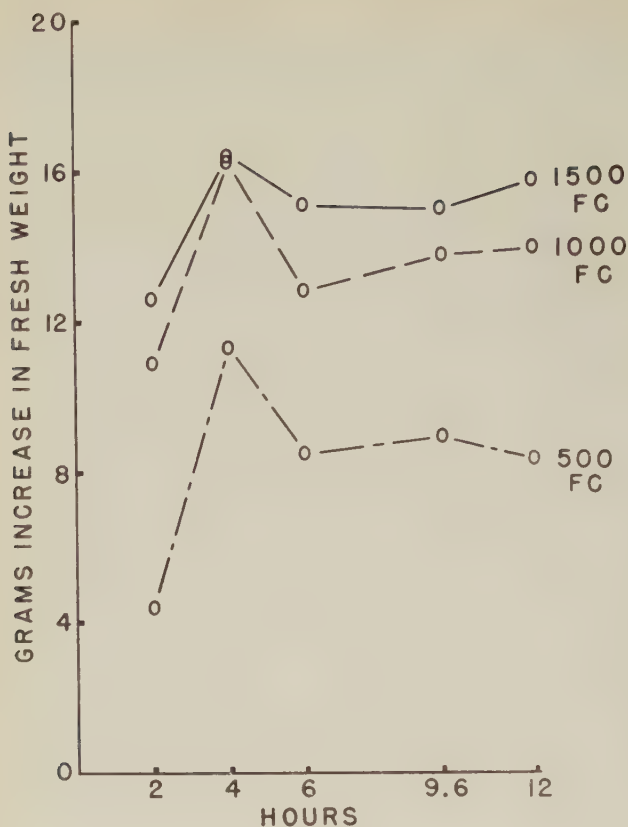


Figure 2. Average increase in fresh weight in 17 days of tops of tomato plants grown under various cycles of light and darkness.

4-hour cycle, markedly less in the 2-hour and 6-hour cycles, and somewhat less in the 9.6 and 12-hour cycles. The increase in dry weight at the highest intensity in the 4-hour cycle was 2.3 times that in the 2-hour cycle, where the poorest growth took place. Figure 4 shows the increases in dry weight of cocklebur plants subjected for 15 days to the same 2, 4, 9.6, 12, and 24-hour cycles at the 3 light intensities. With this plant the best growth was found in the 12-hour cycle, with some decrease in the 24-hour cycle and a larger decrease with increasing shortness of the cycles through 9.6, 4, and 2 hours, except at the 1000 fc intensity, where the poorest growth took place in the 4-hour cycle. The reduced weight increase in the 12-hour cycle at 1500 fc resulted from accidental damage to the leaves of the plants during the experimental period. The increase in dry weight in the 12-hour cycle at 1000 fc was 1.5 times as large as the increase in the 4-hour cycle.

Figure 5 shows the amount of increase in dry weight of the tops of cocklebur plants with 5-minute, 15-minute, 1-hour, 4-hour, and 12-hour cycles at

Figure 3. Average increase in dry weight in 17 days of tops of tomato plants grown under various cycles of light and darkness.

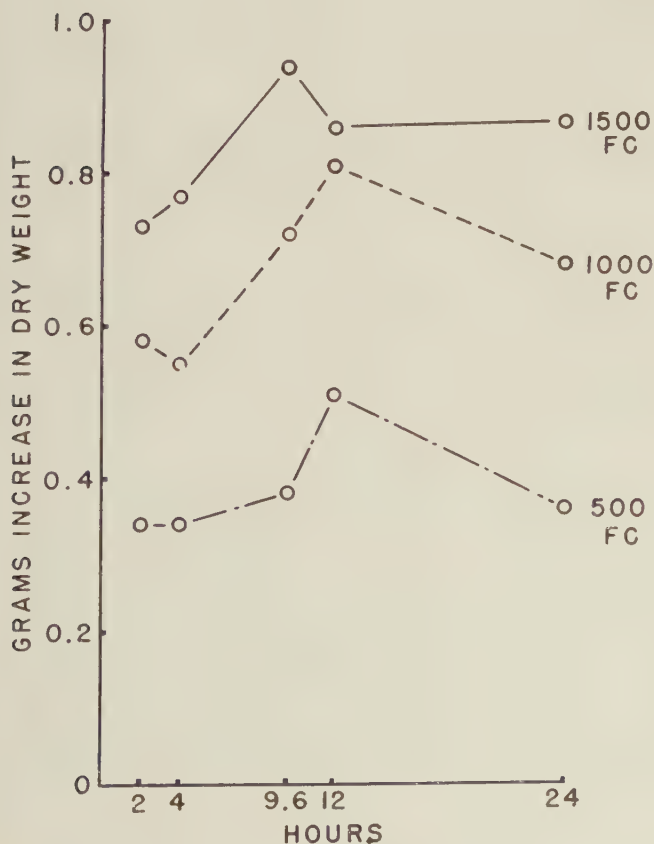
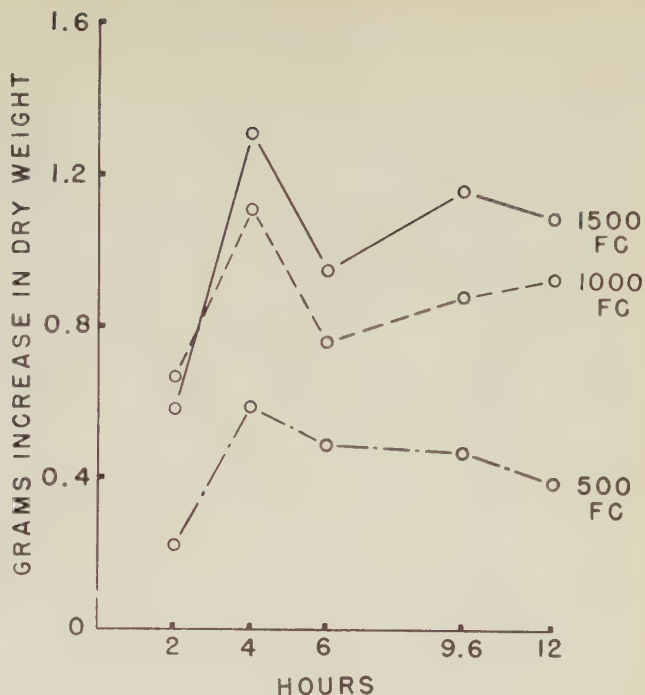


Figure 4. Average increase in dry weight in 15 days of tops of cocklebur plants grown under various cycles of light and darkness. The plants had been induced to flower before being placed under the experimental conditions.

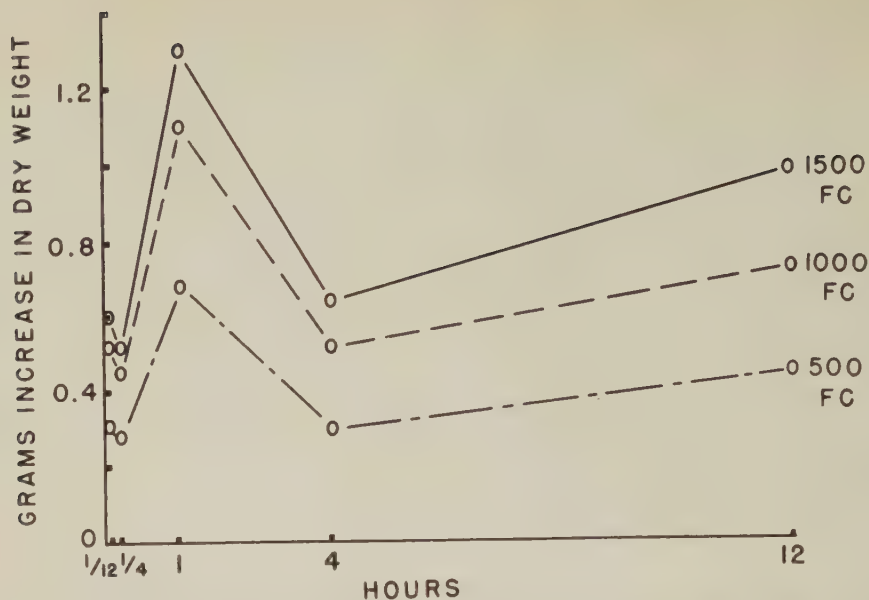


Figure 5. Average increase in dry weight in 20 days of tops of vegetative cocklebur plants grown under various cycles of light and darkness.

the three intensities in 20 days. In this series the greatest growth took place with the 1-hour cycle and somewhat less with the 12-hour cycle. Growth was comparatively poor with the 5-minute, 15-minute, and 4-hour cycles. At 1500 fc the increase in dry weight in the 1-hour cycle was 2.5 times as great as that in the 15 minute cycle.

Plants grown in the shortest cycles began to show an effect on the amount of chlorophyll in the leaves shortly after being introduced into the light boxes. As shown in Figure 6 with a series consisting of 5-minute, 15-minute, 1-hour, 4-hour, and 12-hour cycles at 1500 fc, the amount of chlorophyll in cocklebur leaf samples varied considerably. The 5-minute and 15-minute leaves in this case had one-quarter to one-third as much chlorophyll as the leaves from plants in the longer cycles, and in appearance the leaves of these plants were light green in color and somewhat curled. The effects were equally noticeable at all three intensities.

The cocklebur plants of Figure 4 showed small flower buds at the beginning of the 15-day light-box treatment. Figure 7 shows the effect of the 2, 4, 9.6, 12, and 24-hour cycles on the subsequent development of these buds during the experimental period, as indicated by the total fresh weight of the inflorescences of each group of 9 plants at the time of harvest. The inflores-

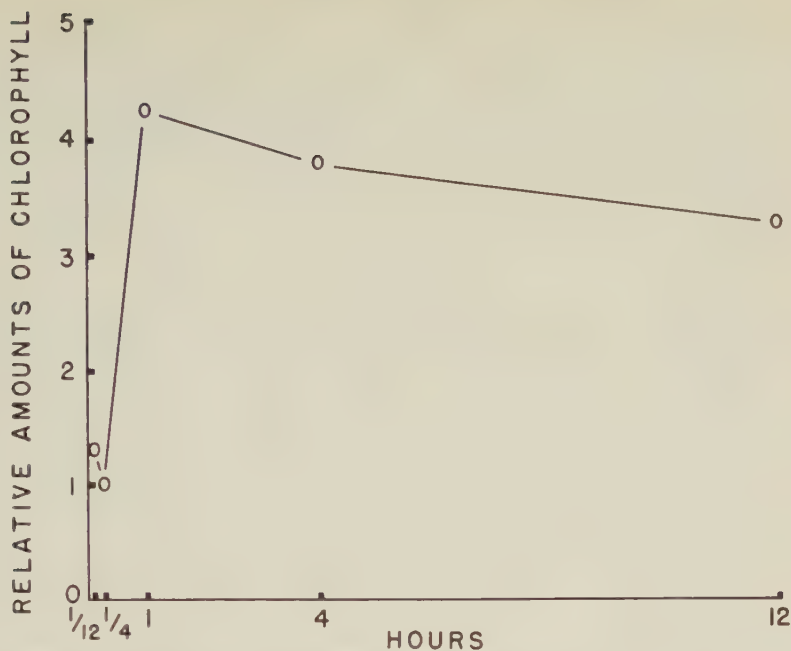


Figure 6. *Effects of various cycles of light and darkness on the amount of chlorophyll in leaves of vegetative cocklebur plants after 20 days, as indicated by relative concentrations of leaf extracts.*

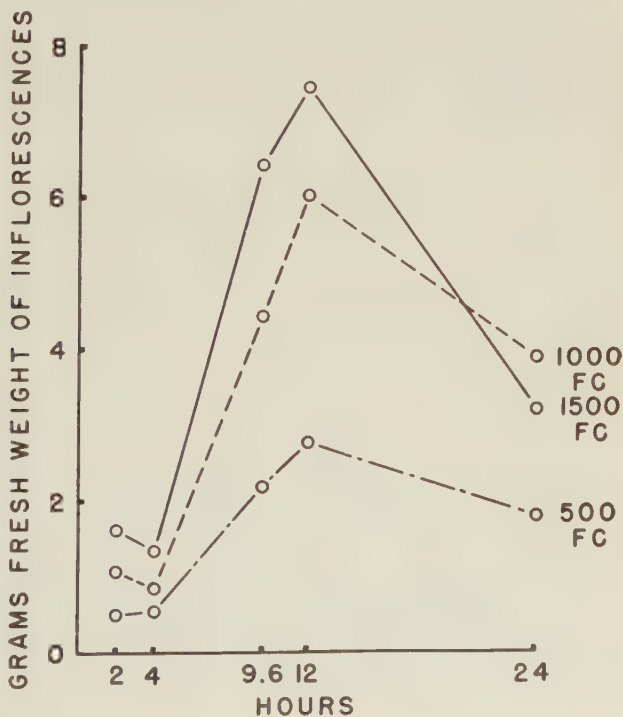


Figure 7. *Effects of various cycles of light and darkness on the fresh weight of inflorescences of induced cocklebur plants after 15 days. Each point represents the total weight of the inflorescences of 9 plants.*

cences from the 12-hour plants at 1500 fc were 5.7 times as heavy as those in the 4-hour cycle and more than twice as heavy as those in the 24-hour cycle. The shapes of the curves correspond well with those of the vegetative growth of the tops. The inflorescences were heaviest at the highest light intensity except in the 24-hour cycle, where the weight fell below that of the ones at 1000 fc.

Discussion

Figure 8 shows a comparison of the growth of plants of *Fagopyrum vulgare* and *Cosmos sulphureus* under various cycles, as drawn from data of Garner and Allard (2). The buckwheat is indifferent to daylength in its flowering response and was in flower at the end of the 34-day test, while the Cosmos is a short-day plant and did not flower under any of the conditions in the 42 days of the test. Both of these plants showed the best vegetative growth, as measured by increase in dry weight of the tops, at the longest (12-hour) and shortest (5-second) time periods employed. The buckwheat grew best in the 5-second cycle of the 5-second, 15-second, 1-minute, 5-minute, 15-minute, 30-minute, 1-hour, and 12-hour cycles employed, and second best in the 12-hour »control» cycle, whereas the Cosmos produced 3.5 times as much dry weight in the 12-hour cycle as in the next best 5-second cycle. With the buckwheat the poorest growth was shown in the 1-minute and 15-minute cycles, with the intermediate 5-minute cycle somewhat better, while the Cosmos produced the smallest amount of dry weight in the 1-minute and 5-minute cycles. Eighty-eight times as much dry matter was produced in the 12-hour cycle as in the 1-minute, and 26 times as much in the 5-second as in the 1-minute.

With the use of plants several weeks old for relatively short experimental periods, it was found in the present study with the cocklebur that the maximum growth as indicated by dry weight increase occurred in the 1-hour cycle, with a minimum in the 15-minute cycle. A second maximum appeared in the 12-hour cycle, with the 4-hour cycle also being comparatively unfavorable for growth and the 24-hour cycle somewhat less so. This growth pattern does not compare well with that found by Garner and Allard for the short-day Cosmos, wherever the same cycles were employed. With the Cosmos the maximum growth took place in the longest 12-hour cycle and the second-best in the shortest 5-second cycle, with a small peak in-between in the 15-minute cycle. The curves for buckwheat and Cosmos are quite comparable.

The possibility that the inhibition of growth of the tomato and cocklebur plants under certain cycles of light and darkness is due to effects on the

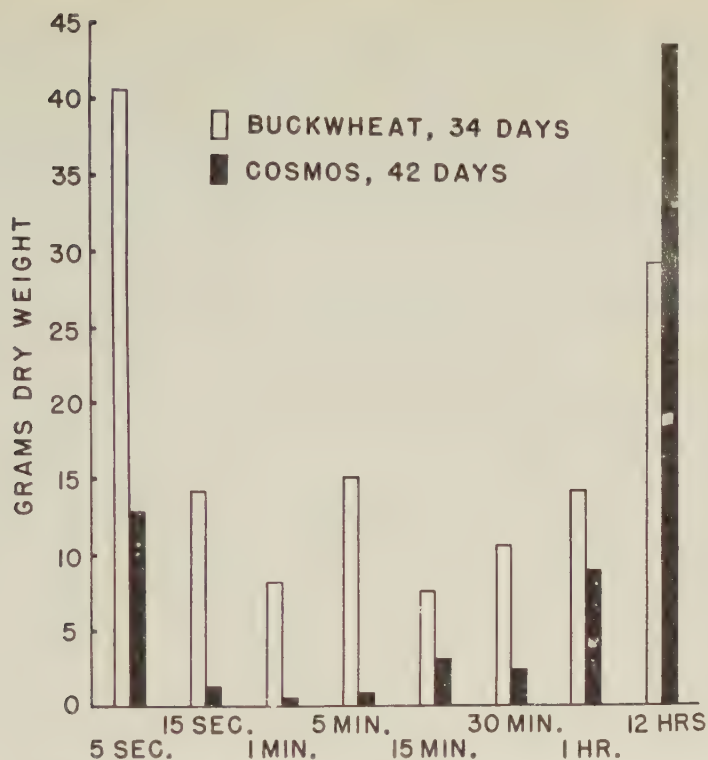


Figure 8. Dry weights of tops of 10 plants in each case of buckwheat and *Cosmos* grown from the seedling stage under various cycles of light and darkness at light intensities between 2000 and 4000 foot-candles. Drawn from data of Garner and Allard (2).

photosynthetic mechanism does not seem very likely in that all of the plants under the various cycles received the same total amount of light during the experimental period. In addition, the growth responses were essentially similar in comparative amount among the different cycles at the 3 light intensities employed, at the highest of which the plants were light-saturated.

Bünning (1) has presented a hypothesis that an endogenous daily rhythm exists in plants which determines their responses to environmental factors. According to this hypothesis, in the course of the 24-hour day the plant goes through one phase (the photophile) during which time light promotes certain activities, while in a subsequent phase (the skotophile) light has no promoting effect or may be inhibitory. Highkin and Hanson (2) suggest an application of this hypothesis to their results in which tomato growth was found to be greatly inhibited in alternating periods of light and dark 6 hours in length and 24 hours in length. Under both these conditions the plants

received light during the skotophile half of a 24-hour period (the normal dark period), and as a result growth was hindered. This hypothesis of Bünning offers a means of explaining inhibition of growth with 6-hour and 24-hour cycles, perhaps, but sheds little light on the reasons for the growth patterns found in the shorter cycles where growth is in some cases much better than in 12-hour cycles. However, the patterns may indicate that other rhythms than diurnal ones are effective. The results with the tomato in the present work indicate that over a short period of time, at least, growth is particularly favored by 4-hour and 12-hour alternations of light and dark, and less favored by cycles in between. With the cocklebur growth is particularly favored by the 5-minute, 1-hour and 12-hour alternations of the ones employed in this study. Possibly different processes taking part in growth respond to cycles of different lengths.

Chlorophyll deficiencies developed in the 5-minute and 15-minute cycles in the cocklebur but not in the other cycles. This agrees with the fact that the poorest cocklebur growth was found in the 15-minute cycles. Garner and Allard found that buckwheat plants become etiolated in the 5, 15, and 30-minute cycles and Cosmos in the 1, 5, and 15-minute cycles.

The effects of various cycles on the growth of inflorescences of induced cocklebur plants, as seen in Figure 7, is very striking, and here the 12-hour cycle is greatly superior to the others. The effect of shorter cycles on inflorescence growth was not studied. No macroscopic flower buds were visible on any of the initially non-induced cocklebur plants at the end of the experimental periods. In noting the time required for flowering of the various plants tested under the different cycles, Garner and Allard found that the photoneutral buckwheat plants bloomed at the same time under all the cycles. The long-day *Rudbeckia bicolor*, however, grown under 5-second, 15-second, 1-minute, 5-minute, 30-minute, and 1-hour cycles, flowered most rapidly in the 5-second and 15-second cycles; and the long-day *Delphinium ajacis* also flowered most rapidly in the 5-second and 15-second cycles. Flowering data for short-day plants were not recorded.

Summary

1. Young tomato plants were grown under various cycles of alternating light and dark periods of equal length (each period 2, 4, 6, 9.6, or 12 hours in length) for 17 days. Growth as measured by increase in height, fresh weight, and dry weight was greatest under cycles of 4 hours of light and 4 hours of darkness. Growth was next best under 12-hour cycles, and the poorest growth took place under 2-hour cycles.

2. In young cocklebur plants grown for 2 to 3 weeks under 5-minute, 15-minute, 1-hour, 2-hour, 4-hour, 9.6-hour, 12-hour, and 24-hour cycles the most growth took place under cycles of 1 hour of light and 1 hour of darkness, with the 12-hour cycle being less favorable for growth but still better than the other cycles. Poorest growth took place under the 15-minute cycle.

3. The growth patterns among the various cycles were not affected by three different light intensities of 500, 1000, and 1500 foot-candles, although the best growth occurred at the highest intensity.

4. Leaves of cocklebur plants grown under 5-minute and 15-minute cycles were light green in color and contained one-fourth to one-third as much chlorophyll as leaves from plants under longer cycles.

5. The growth of the inflorescences of induced cocklebur plants was greatly decreased under cycles longer and shorter than 12-hour cycles.

6. The results suggest that other rhythms than diurnal ones are operative in the growth of the tomato and the cocklebur.

The author wishes to thank Dr. F. W. Went for his kind help and advice and for the opportunity to use the facilities of the Earhart Laboratory of the California Institute of Technology. The work reported here was done while the author was a Fellow of the National Science Foundation.

Literature

1. Bünning, E.: Über die photophile und skotophile Phase der endogenen Tagesrhythmik. — *Planta* 38: 521. 1950.
2. Garner, W. W. & Allard, H. A.: Effect of abnormally long and short alternations of light and darkness on growth and development of plants. — *Jour. Agr. Res.* 42: 629. 1931.
3. Highkin, H. R. & Hanson, J. B.: Possible interaction between light-dark cycles and endogenous daily rhythms on the growth of tomato plants. — *Plant Physiol.* 29: 201. 1954.

The Assimilation of Ammonia and Nitrate by Nitrogen-Starved Cells of *Chlorella vulgaris*

I. The Assimilation of Small Quantities of Nitrogen

By

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(Received August 8, 1955)

Introduction

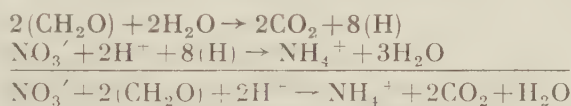
Plants grown with nitrate-N as a nitrogen source often differ considerably in composition and metabolism from similar plants grown in ammonium-N (see reviews by Nightingale (1937) and Street (1949) but our knowledge of the processes underlying these differences is limited. In this, and the following papers, work on the assimilation of nitrate and ammonium-N by nitrogen-starved cells of *Chlorella vulgaris* is described which illustrates how metabolism is altered by the form in which nitrogen is supplied.

Nitrogen-starved organisms assimilate added nitrogen rapidly and, when respiration has been measured, a higher rate has been found to accompany the nitrogen assimilation: Syrett (1953 b) and Yemm and Folkes (1954) cite relevant papers. Fewer workers have compared ammonium-N and nitrate-N assimilation. Willis and Yemm (1955) have recently described the changes in oxygen absorption and carbon dioxide production which follow the addition of ammonium-N, hydroxylamine-N, nitrite-N and nitrate-N to nitrogen-starved barley roots. Their results have much in common with those described here.

Syrett (1953 a) showed that nitrogen-starved cells of *Chlorella* assimilate small quantities of ammonium-N rapidly, the assimilation being accompanied by increased rates of oxygen uptake and carbon dioxide production which

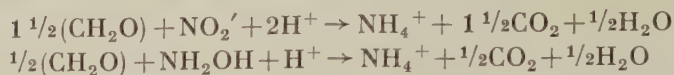
continue until all the ammonium-N has been assimilated. Syrett (1953 b) and Yemm and Folkes (1954) have discussed possible explanations of the respiratory stimulation.

When nitrate-N is added to nitrogen-starved cells the rate of carbon dioxide output also increases greatly; oxygen uptake increases to a lesser extent. Now the quantitative differences which one would expect between the gas exchanges accompanying ammonium-N and nitrate-N assimilation by nitrogen-starved *Chlorella* can be deduced from the fundamental work of Warburg and Negelein (1920). These workers suspended *Chlorella* cells in a mixture of 0.01 *M* HNO₃ and 0.1 *M* NaNO₃ with a pH of 2. They found that nitrate was reduced to ammonia which accumulated in the medium. When nitrate was reduced, an 'extra' carbon dioxide production was superimposed on the basic respiration (which had a R.Q. of unity) and they found that two molecules of 'extra' carbon dioxide were produced for each nitrate ion which was reduced. Such a quantitative relationship is expected if carbohydrate, or some substance of the same degree of reduction, is the carbon substrate from which carbon dioxide is produced by metabolism together with the hydrogen necessary for nitrate reduction: —



When nitrate is added to nitrogen starved cells of *Chlorella* all the nitrogen is assimilated into organic compounds and ammonia does not accumulate as in the experiments of Warburg and Negelein. One would then expect to find that the gas exchange associated with nitrate *reduction* was superimposed on the gas exchange associated with nitrogen *assimilation*. When a known quantity of ammonium-N is added the gas exchange associated with its assimilation can be measured. If the same quantity of nitrate-N is added to a similar sample of cells the gas exchange expected during its assimilation will be that accompanying ammonium-N assimilation plus two molecules of carbon dioxide for each nitrate ion which is assimilated. Thus the production of 44.3 cu.mm. of 'extra' carbon dioxide is expected for each nitrate ion which is assimilated but the total volume of oxygen consumed should be the same whether ammonium or nitrate-N is added. The finding of such a quantitative relationship does not necessarily imply that ammonia is an intermediate of nitrate assimilation as Burström (1945) has clearly pointed out. This quantitative relationship must apply if carbohydrate, or some substance of the same degree of reduction, is the intracellular carbon source and if the products of nitrate-N and ammonium-N are the same; this is true whatever the path of nitrate reduction.

Similar equations to the one above, can be written for the gas exchange expected to accompany the reduction of nitrite-N and hydroxylamine-N: —



Kessler (1953) has already shown that the expected gas exchange is found when nitrite is assimilated by nitrogen starved cells of *Ankistrodesmus*.

Experimental part

Chlorella vulgaris (Pearsall's strain) was used. The culture conditions and method of nitrogen starvation are described in the following paper.

Gas exchange was measured by the conventional Warburg technique in darkness and at 25° C. Oxygen uptake was obtained from flasks with caustic potash in the centre well and carbon dioxide production from the differences between these flasks and similar ones without caustic potash. The suspending medium for the experiments was 0.067 *M* phosphate with 0.0017 *M* magnesium sulphate, pH 6.0. A correction for carbon dioxide retention was applied (Umbreit, Burris and Stauffer, 1949); experiments in which acid was added to the medium at the end of the experiment to liberate retained carbon dioxide showed that this correction was adequate when only small quantities of ammonium-N, nitrite-N or nitrate-N were assimilated. Two microatoms of nitrogen as ammonium sulphate, potassium nitrate, sodium nitrite or hydroxylamine hydrochloride were added to each flask; all salts were dissolved in the suspending medium. Experiments were done in duplicate and the mean values plotted.

The results of one experiment are shown in Figure 1 and those of a second in Table 1.

Discussion

The addition of any of the nitrogen sources is immediately followed by an increase of the rate of gaseous exchange. Both oxygen uptake and carbon dioxide production increase greatly when ammonium-N is added but the rate of oxygen uptake increases more and the R.Q. falls from its initial value of about 1.1 to 0.8. The low R.Q. is presumably correlated with the conversion of carbohydrate to more oxidized nitrogenous compounds (Syrett 1953 b). Yemm and Folkes (1954) observed a similar low R.Q. when yeast assimilated ammonium-N. The addition of both nitrite and nitrate is followed by a much larger increase of the rate of carbon dioxide production than of that of oxygen uptake and consequently a high R.Q. accompanies assimilation. A high R.Q. accompanying nitrate assimilation has frequently been observed (Burström 1945, Willis and Yemm 1955) and can be attributed to the pro-

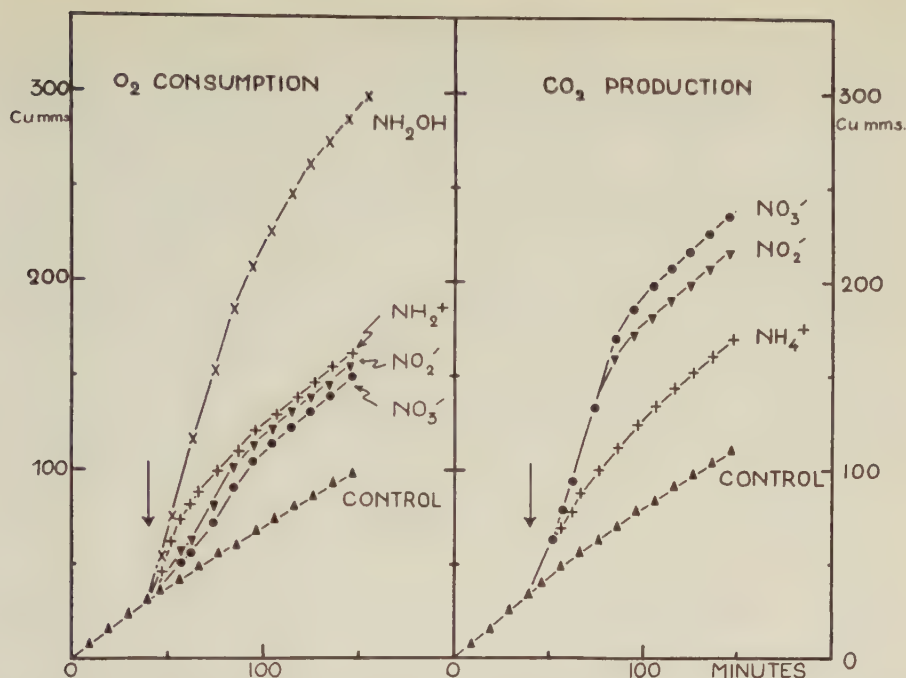


Figure 1. Gas exchange following the addition of 2 μ -atoms nitrogen to nitrogen-starved *Chlorella*. Each flask contained 15.0 mg. dry wt. cells, pH 5.9, 0.067 M phosphate.

Correction: in left diagram instead of NH_2^+ read NH_4^+ .

duction of 'extra' carbon dioxide which accompanies nitrate reduction. It is interesting that the maximum rate of carbon dioxide production is the same whichever nitrogen source is added. This suggests that some process connected with decarboxylation may be limiting in these cells.

It is not easy to decide how to calculate the total gas exchange associated with assimilation since there is doubt whether one should allow for a basic

Table 1. Experiment 2. The gas exchange following the addition of ammonia, nitrite and nitrate to nitrogen-starved cells of *Chlorella*.

Addition (2 μ moles)	Total gas exchange (μ l.)		'Extra' CO_2 (μ l.)	'Extra CO_2 N added (μ moles) (μ g. atoms)	Maximum rate of gas exchange (μ l./10 min.)		R. Q.
	O_2	CO_2			O_2	CO_2	
NH_4^+	— 73	71	—	—	— 26.7	21.7	0.81
NO_2^-	— 80	140	69	1.54	— 10.0	21.3	2.13
NO_3^-	— 71	166	95	2.12	— 9.0	23.0	2.47

Each flask contained 13.5 mg. dry weight cells.

metabolism during the period of assimilation or not. However, if one assumes that all the gaseous exchange during the period of rapid metabolism is associated with nitrogen assimilation the volumes of oxygen consumed and carbon dioxide produced are fairly close to those expected from the argument outlined above. Figure 1 shows that in experiment 1, except for hydroxylamine, the total volume of oxygen consumed was finally much the same whichever nitrogen source was added although the rate of uptake was much greater when ammonium-N was added. Table 1 gives a similar result for the second experiment. In experiment 1 the amount of 'extra' carbon dioxide produced is 1.41 molecules for each nitrite ion assimilated and 1.90 molecules for each nitrate ion; values for experiment 2 are shown in Table 1. These values agree well, in general, with the calculated values of 1.5 and 2.0.

Hydroxylamine gives anomalous results. According to the argument above the total volume of oxygen taken up when this is added, should be the same as that taken up when the same quantity of ammonium-N is assimilated and the 'extra' carbon dioxide should be 0.5 molecule per N-atom assimilated. Clearly the volume of oxygen taken up is much larger than expected. The volume of carbon dioxide produced was not determined in this experiment but in other experiments it is found to be a little greater than the volume of oxygen consumed. The respiration rate is thus greatly stimulated by the addition of hydroxylamine but this stimulation does not appear to be associated with the normal incorporation of nitrogen into cell substance as it is with the other nitrogen sources. Analysis of the medium shows that the added hydroxylamine has disappeared when the respiration rate has slowed down and the cells show an increase of soluble organic nitrogen. These results could be explained if low concentrations of hydroxylamine partially uncouple synthesis from respiration like 2,4-dinitrophenol. Willis and Yemm (1955) found that hydroxylamine stimulated the oxygen uptake of nitrogen starved barley roots but this initial stimulation was followed by a decrease of the rate to below the control level. However, their concentration of hydroxylamine was three times that used in these experiments with *Chlorella*.

Summary

The rapid assimilation of small quantities of ammonium, nitrite and nitrate-N by nitrogen-starved cells of *Chlorella vulgaris* is accompanied by high rates of gas exchange. When all the added nitrogen has been assimilated the rates of gas exchange return to the control values. The *total volume* of oxygen taken up during the period of nitrogen assimilation is about the same whichever nitrogen source is added but the *rate* is highest with ammonium-N

and lowest with nitrate-N. On the other hand, the *rate* of carbon dioxide production is much the same whichever salt is added but the *total volume* of carbon dioxide produced is greatest when nitrate-N is added and least with ammonium-N.

The quantities of oxygen absorbed and carbon dioxide produced agree with those expected if ammonium, nitrite and nitrate-N are all assimilated to the same final products and if carbohydrate is the carbon source in the cells.

A high respiration rate also follows the addition of hydroxylamine but the gas exchange differs markedly from that expected if hydroxylamine were assimilated to the same products as the other nitrogen sources.

The respiration determinations were made with a Warburg apparatus purchased with a grant from the Dixon Fund of the University of London.

References

- Burström, H.: The nitrate nutrition of plants. — *Lantbr.högsk. Ann.* 13: 1. 1945.
- Kessler, E.: Über den Mechanismus der Nitratreduktion von Grünalgen. — *Flora* 140: 1. 1953.
- Nightingale, G. T.: The nitrogen nutrition of green plants. — *Bot. Rev.* 3: 85. 1937.
- Street, H. E.: Nitrogen metabolism of higher plants. — *Advanc. Enzymol.* 9: 391. 1949.
- Syrett, P. J.: The assimilation of ammonia by nitrogen-starved cells of *Chlorella vulgaris*. Part I. The correlation of assimilation with respiration. — *Ann. Bot. N.S.* 17: 1. 1953 a.
- The assimilation of ammonia by nitrogen-starved cells of *Chlorella vulgaris*. Part II. The assimilation of ammonia to other compounds. — *Ann. Bot. N.S.* 17: 21. 1953 b.
- Umbreit, W.W., Burris, R. H. & Stauffer, J. F.: *Manometric technique and tissue metabolism*, p. 19. — Minneapolis: Burgess Publishing Co. 1949.
- Warburg, O. & Negelein, E.: Über die Reduktion der Salpetersäure in grünen Zellen. — *Biochem. Z.* 110: 66. 1920.
- Willis, A. J. & Yemm, E. W.: The respiration of barley plants. VIII. Nitrogen assimilation and the respiration of the root system. — *New Phytol.* 54: 163. 1955.
- Yemm, E. W. & Folkes, B. F.: The regulation of respiration during the assimilation of nitrogen in *Torulopsis utilis*. — *Biochem. J.* 57: 495. 1954.

The Determination of Paths of Air Movement in Leaves

By

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(Received August 10, 1955)

Introduction

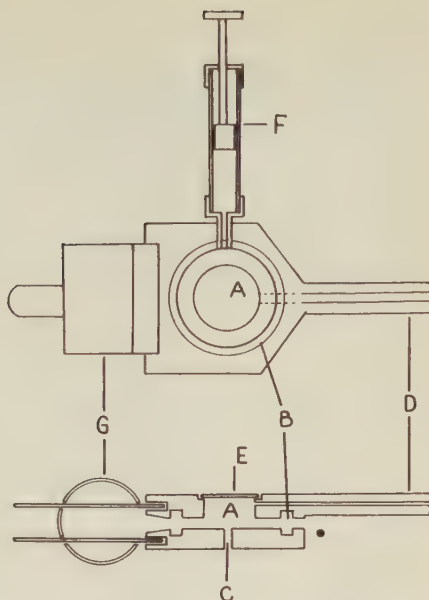
A knowledge of the paths of air movement available in a leaf is of importance for the study of leaves as organs of transpiration as well as for investigations involving airflow porometers, Darwin and Pertz (1911), Gregory and Pearse (1934), Heath and Russell (1951), Knight (1915). Porometer results may be affected by changes in the airspace system of leaves, brought about by changes in the leaf water content, even when stomatal conductivities remain constant (Meidner 1955). Two simple instruments will be described, both of which were designed to test qualitatively for paths of airflow in leaves when small pressure differences were maintained between the outside and the inside of a leaf.

The three main paths of air movement in leaves

1. Air may move more or less directly from one epidermis to the other via stomata in a vertical direction through the mesophyll; this is the case in some amphistomatous leaves.
2. Air may move into the leaf via stomata in one epidermal area, then laterally through the mesophyll between the two epidermes and out of the leaf via stomata situated in an adjoining area of epidermis; this is the case with some hypostomatous leaves.
3. Air may move both by paths 1 and 2 described above; this is the case with some amphistomatous leaves.

Figure 1. *The leaf membrane chamber in plan and section.*

- A — Chamber
- B — Groove for Washers
- C — Air inlet Vent
- D — Attachment Tube for Suction
- E — Observation Window
- F — Syringe
- G — Spring Steel Clip



Apparatus

A. *The Leaf Membrane Chamber*

Construction. The design of the instrument is shown in diagram in Figure 1. The chamber was constructed from perspex sheets 5.0 mm. thick. The microscope cover glass E served as observation window. The spring steel clamp G held together the two parts forming the chamber. The radius of the circular chamber A was 7.5 mm. It could be filled with water by means of the syringe F. The width of groove B, concentric with the wall of the chamber, was 2.5 mm. and its radii were 11.0 mm. and 13.5 mm. In the lower portion of the chamber two kinds of gelatine or latex washers could be used. If a ring washer was used the air inlet hole C remained open and if a plate washer was used the air inlet hole C was closed.

Method of use. A leaf to be tested was placed between the washers of the chamber, which was then closed by means of the spring steel clamp and filled with water from syringe F. Either a porometer or a gentle suction pump was connected to the air outlet tube D. During a test the leaf membrane chamber had to be held with the air outlet tube D pointing diagonally upwards so that the stream of bubbles escaping from the leaf could be observed as it passed through the water.

By using two ring washers in the leaf membrane chamber and applying vaseline to the portion of the leaf which remained outside the chamber,

path 1 was tested for. If the plate washer was used in the lower portion of the chamber, path 2 was tested for, provided the part of the leaf which remained outside the chamber remained free of vaseline. If both paths 1 and 2 were available in a leaf it was possible to judge which was the main path for air movement by comparing the streams of bubbles during the two separate tests.

Leaves to be tested in the leaf membrane chamber must have their stomata open, a condition easily ascertained with the aid of a porometer.

B. *The Double Bore Porometer*

Construction. The instrument is shown in diagram in Figure 2. It consisted of the attachment clamp C fitted to the double bore capillary tube A. The attachment clamp C consisted of the perspex jaws H and the spring steel clip E. The upper jaw of the clamp had two parallel and vertical holes, whereas the lower jaw had, besides two holes corresponding to those in the upper jaw, the single diagonally placed air inlet vent F which was closed by the washer for use with amphistomatous leaves but left open by the special washer for use with hypostomatous leaves.

The two mercury columns M' and M'' descended in the capillary bores, but did not spill on account of the small size orifices of the air escape vents D' and D''.

The gelatine washers used were of several kinds depending on the nature of the leaves to be tested: —

For amphistomatous leaves a lower circular plate washer with two small holes was used as shown in Fig. 2 a. The two holes in the washer were cut diagonally and their entrances were either 1.0 mm. or 0.5 mm. apart. This washer closed the air inlet hole F in the lower portion of the clamp. The upper washer had only one eccentrically placed hole which fitted immediately above one of the holes in the lower washer. To insure proper registration of the washers two guide keys B' and B'' were provided for each washer.

For hypostomatous leaves an upper »blind» washer without any hole and a specially shaped lower washer were used as shown in Fig. 2 b. These washers insured that the only tested air movement within the leaf was across the strip of tissue either 1.0 mm. or 0.5 mm. wide.

Method of Use. The two mercury columns M' and M'' were raised to the top of the capillary tubes by applying a rubber bulb at vents D' and D'' or simply by inverting the instrument.

After the leaf to be investigated was placed between the washers, the descent of the mercury columns was timed with a stop watch. (As a double bore stop-cock was not available for fitting at the top of the capillary tubes,

Figure 2. The double bore porometer in section.

- A — Double Bore Capillary Tube
- B', B'' — Guide Keys for Washers
- C — Attachment Clamp
- D', D'' — Air Escape Vents
- E — Steel Clip
- F — Air Inlet Vent
- M', M'' — Mercury Columns
- G — Washers for use with amphistomatous leaves

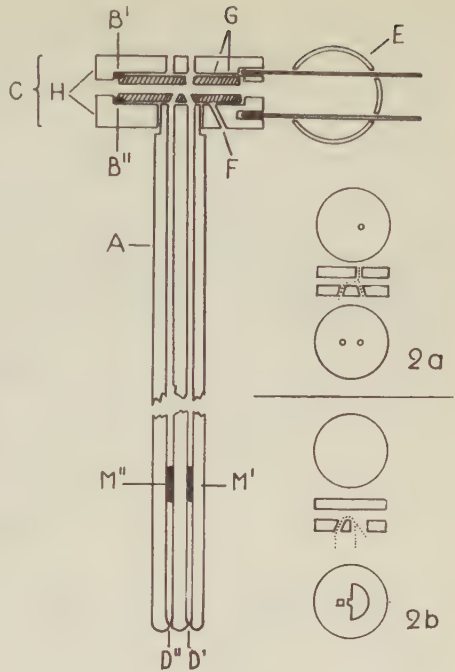


Figure 2 a. Upper and lower washer in plan and section for use with amphistomatous leaves. The dotted lines represent possible paths of air movement.

Figure 2 b. Upper and lower washer in plan and section for use with hypostomatous leaves. The dotted lines represent possible paths of air movement.

the mercury was prevented from descending until required, by holding a pad of gelatine against the two escape vents D' and D'').

With a leaf which did not allow for lateral air movement, only one of the mercury columns descended while the other remained stationary. With a leaf which put the two capillary tubes of the double bore porometer into communication via the mesophyll air space system, both mercury columns M' and M'' descended. From the rates of descent, if not equal, a measure was obtained of the degree of air movement in the lateral direction across a strip of tissue 1.0 mm. or 0.5 mm. wide.

In the case of hypostomatous leaves the mercury column M' descended at its maximum rate because it was in direct communication with the atmosphere, whereas column M'' descended at a rate which depended on the conductivity of the stomata and on the resistance offered by the strip of tissue 1.0 mm. or 0.5 mm. wide.

The use of the double bore porometer gave more detailed information than the leaf membrane chamber; because in the latter the limiting factor was the width of the gelatine washer, which could not be narrowed down to less than 2.5 mm., whereas in the double bore porometer the distance between the entrance holes to the two capillary bores could be as little as 0.5 mm.

Table 1. *Habitat, thickness, stomatal numbers, stomatal pore areas per square millimeter and mesophyll resistance of six hypostomatous leaves.*

Species	Habitat	Leaf Thickness μ	Stomatal Number per mm^2	Total Stomatal Pore Area per $\text{mm}^2 \mu^2$	Resistance of mesophyll tissue in Gregory and Pearse units.
<i>Psychotria capensis</i> ...	Deep shade	362	160	1120	0.6— 0.8
<i>Scolopia mundii</i>	shade	322	170	2040	1.6— 2.5
<i>Canthium spinosum</i> ...	shade	255	230	3200	7.0—12.0
<i>Mangifera indica</i>	open veld	244	500	3500	above 60.0 no lateral airmovement
<i>Dalbergia obovata</i> ...	sunny forest margin	222	200	3600	above 60.0 no lateral airmovement
<i>Chaetacme aristata</i> ...	open veld	310	400	4000	above 60.0 no lateral airmovement

Results

With the instruments described, supplemented by anatomical studies, the leaf of *Erythrina caffra* was found to be constructed in such a way that air movement took place only in the vertical direction i.e. through air spaces which were »capped» with stomata, either at one or both ends. The resistance offered to lateral movement of air was practically infinite.

Neger's (1918) description of leaves composed of small islets of mesophyll which appeared »hermetisch gegeneinander abgeschlossen» seemed applicable in this case. The results also indicated one of the physiological implications of Wylie's (1952) observations on bundle sheath extensions in leaves of dicotyledons.

Also some hypostomatous leaves investigated with the two instruments were found *not* to allow for lateral movement of air over distances of 0.5 mm. From the data in Table 1 it appears that there exists a relationship between the habitat, the total stomatal pore area per square millimeter, and the degree of continuity of the air space system of leaves. The latter was measured quantitatively by a method described by Meidner (1955) and is expressed as the resistance of the mesophyll tissue.

Summary and Conclusions

Two instruments have been described with which it is possible to assess qualitatively the paths of air movement available in a leaf. Results have been obtained with several leaves which indicate that the air space systems are not continuous in all leaves and that the degree of continuity of the air space

system appears to be related to the total stomatal pore area per square millimeter as well as to the habitat of a plant.

References

- Darwin, F. & Pertz, D. F. M.: On a new method of estimating the aperture of stomata. — *Proc. Roy. Soc. B.* 84: 136. 1911.
- Gregory, F. G. & Pearse, H. L.: The resistance porometer and its application to the study of stomatal movements. — *Proc. Roy. Soc. B.* 114: 477. 1934.
- Heath, O. V. S. & Russell, J.: The Wheatstone Bridge porometer. — *J. Exp. Bot.* 2: 111. 1951.
- Knight, R. C.: A convenient modification of the porometer. — *New Phytol.* 14: 212. 1915.
- Meidner, H.: Changes in the resistance of the mesophyll tissue with changes in the leaf water content. — *J. Exp. Bot.* 6: 94. 1955.
- Neger, F. W.: Die Wegsamkeit der Laubblätter für Gase. — *Flora* 111: 112. 1918.
- Wylie, R. B.: The bundle sheath extensions in leaves of dicotyledons. — *Am. J. Bot.* 39: 645. 1952.

Paper Electrophoresis Patterns of Enzymes Involved in Polyglucoside Synthesis in *Oscillatoria Princeps* and Its Low Temperature Strains

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Introduction

The techniques of paper partition chromatography and paper electrophoresis for the study of genetically different tissues have recently found application in the field of genetic studies. They have proven to be valuable tools for the detection of genotypic differences in otherwise morphologically identical tissues.

The use of paper chromatography for the detection of genic changes, was introduced by Hadorn and Mitchell (1951). The technique has been used with success by Buzzati-Traverso (1953) for the detection of single gene differences in strains of *Drosophila melanogaster* and in plants.

Paper electrophoresis is the migration of substances on the surface of paper under the influence of an applied electrical potential. Haugaard and Kroner (1948) first reported the use of the technique for the separation of the amino acids. The separation of enzymes by this method has been reported by Mills and Smith (1951). They were able to resolve glucuronidase, the phosphatases and esterases. Reid (1950), was able to resolve polygalacturonase and amylase into two components, and the fungal amylases into four via these techniques of paper partition.

These methods should prove of considerable interest in the detection of differences between enzymes having the same properties and functions in different species. The techniques seemed to hold promise for the resolution of the phosphorylating enzymes of wild-type and variant, or mutant strains of organisms.

With this in mind, it was decided to attempt an electrophoretic-chromatographic study of these enzymes in normal and low-temperature variant *Oscillatoria princeps*.

The enzymes involved in polyglucoside synthesis have been investigated in this blue-green alga, and their properties delineated in a series of papers by Fredrick (1951, 1952, 1953, 1954, 1955), and Fredrick and Mulligan (1955). It was found that two distinct enzymes were involved in the synthesis of polyglucoside from glucose-1-phosphate: a *phosphorylase* which synthesized α -1 : 4 linkages between glucosidic residues, and a *branching enzyme* which caused the synthesis of α -1 : 6 linkages, and hence, was responsible for branching in the sugar.

Particular attention was devoted to the study of these enzymes in a variant strain of *Oscillatoria princeps*, induced by the growth of cultures of normal *Oscillatoria* at low temperatures (Fredrick 1952, 1953). In this variant, the polyglucoside produced showed evidence of less branching than found in normal *Oscillatoria* sugar.

Fredrick (1954) reported the identity of the enzymes from normal and variant *Oscillatoria princeps*, as far as physical constants were concerned. The similarity in chemical action was reported in a recent paper (Fredrick and Mulligan 1955). As a result of these previous studies, a hypothesis was formulated that the difference in polyglucosides of these two strains, was due to a decrease in absolute concentration of *branching enzyme* in the variant form, which gave rise to a «lag» in the establishment of α -1 : 6 linkages and made for the synthesis of a relatively unbranched, amylose-like polyglucoside (Fredrick 1955).

Since no differences in the properties of the enzymes in normal and low-temperature variant strains of this alga were detected, it was decided to attempt a differentiation by means of paper partition techniques. This study combined the techniques of paper partition chromatography and paper electrophoresis.

Experimental

I. Preparation of Enzymes

A crude extract with sodium bicarbonate was prepared from cultures of normal *Oscillatoria princeps* and from low temperature cultures of this alga, as described

(Fredrick 1951, 1952). These extracts were then subjected to electrophoresis, 0.05 ml. of extract being used for each chromatogram.

Each crude extract was purified by fractional precipitation with ammonium sulfate (Fredrick 1954). The purified enzyme mixtures were also subjected to electrophoretic study.

Isolation of the phosphorylase and the branching enzyme in the purified mixtures described above was accomplished by reprecipitation from 0.1 saturated ammonium sulfate solution (Fredrick and Mulligan 1955). Electrophoretic patterns were determined on each purified enzyme, and later compared.

II. *Electrophoretic Method*

Paper electrophoresis was carried out on 21 cm. \times 3 cm. strips of Whatman No. 1 filter paper. The strips were impregnated with a solution containing 1.4 per cent sodium bicarbonate and 0.1 per cent manganese sulfate, dried in a warm air current and spotted with the enzyme solutions. All runs were made on two parallel strips sandwiched between plate glass coated with silicone grease to minimize capillary effects. The ends of the strips dipped over into two glass vessels containing the buffer-salt mixture with which the strips had been impregnated. A simple full-wave rectifier supplied the difference in potential via carbon electrodes in each vessel. A schematic diagram of this rectifier is shown in Figure 1.

Separations were accomplished at a potential of 4 volts/cm. and 4 milliamperes for 12 hours. The pH of the buffer-salt was 6.8, and the temperature was 23° Centigrade.

Detection of the materials on the finished strips was accomplished by immersing the dried strip in a solution of 0.05 per cent bromphenol blue in 0.5 per cent sulfuric acid at 55° C. for 3—5 minutes. The strip was then washed in warm water for one minute and dried in a hot air current.

III. *Detection of Enzymes*

Two parallel strips were run at one time. One strip was stained and used as a map. The areas were marked off by means of a soft wax pencil on the unstained strip. Each marked area was then cut out and eluted with cold sodium bicarbonate-manganese sulfate buffer at pH 7.2. The resulting fractions were tested for enzyme activity on a buffered substrate of glucose-1-phosphate (Fredrick 1951). The iodine color of the polyglucoside produced was taken as a qualitative indication of the presence or absence of the enzymes (Fredrick 1951, 1952).

IV. *Quantitative Differentiations*

In one experiment, care was taken in the preparation of crude sodium bicarbonate extracts of normal and low-temperature *Oscillatoria princeps*. Volumes of extracting solutions were kept as constant as possible, as was the time of extraction, etc. The extracts were spotted on the filter paper, at a distance of approximately 8 cm. from the cathode end of the strip. After running for 12 hours at a potential of 4 v./cm. and 4 m.a., the paper was dried in a warm air current for 30 minutes. Parallel strips

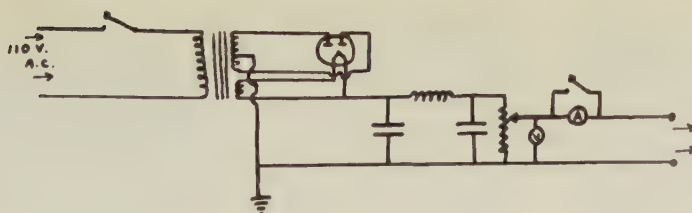


Figure 1. Schematic diagram of the circuit of the full-wave rectifier used in the electrophoresis set-up. The transformer is a 270—0—270 volts, 120 m.a. type. The vacuum tube is a 5U4. The rheostat is a 10,000 ohms, 50 watt, ohmite type.

of normal extract and low-temperature extract were run off at the same time, under the most identical of conditions.

The strips were then immersed for exactly 5 minutes in a dye bath of 0.03 per cent Naphthalene Black 12B.200 in methanol containing 10 per cent acetic acid. The strips were washed in methanol containing 1 per cent mercuric chloride for 2 minutes. The second wash was in methanol alone for 3 minutes. The strips were dried in warm air currents for 30 minutes. Each zone which had exhibited enzymatic activity in the third part of this study was cut out so that the distance from the center of these zones to the blank edge surrounding the zones was constant. The cut-out zones were extracted with a solution of 50 per cent methanol containing 4 per cent sodium carbonate. Each extract was then read on a Coleman Spectrophotometer at 595 m μ . Differences in the readings were taken as indicative of differences in the concentration of the enzyme proteins. This method had been used successfully by Flynn and de Mayo (1951) for the determination of the blood globulins.

Results

It was found that crude extracts of *Oscillatoria princeps* separated into five zones in the normal strain (N), and into six zones in the low-temperature variants (LTV). This can be seen in Figure 2.

The over-all patterns of both N and LTV crude extracts were remarkably the same except for the zone of slow-moving protein exhibited by the LTV extracts. The zones, the distance migrated in 12 hours, and the rates of migration are summarized in Table 1.

In purified preparations, obtained by fractionation with 0.8 saturated ammonium sulfate, the patterns for N and LTV fractions were identical. As can be seen in Figure 3, the zone apparent in the LTV crude extract (Zone II) does not appear in purified preparations of LTV material. It is interesting to note that samples eluted from this chromatogram still gave rise to the synthesis of a less-branched polyglucoside than those eluted from N chromatograms.

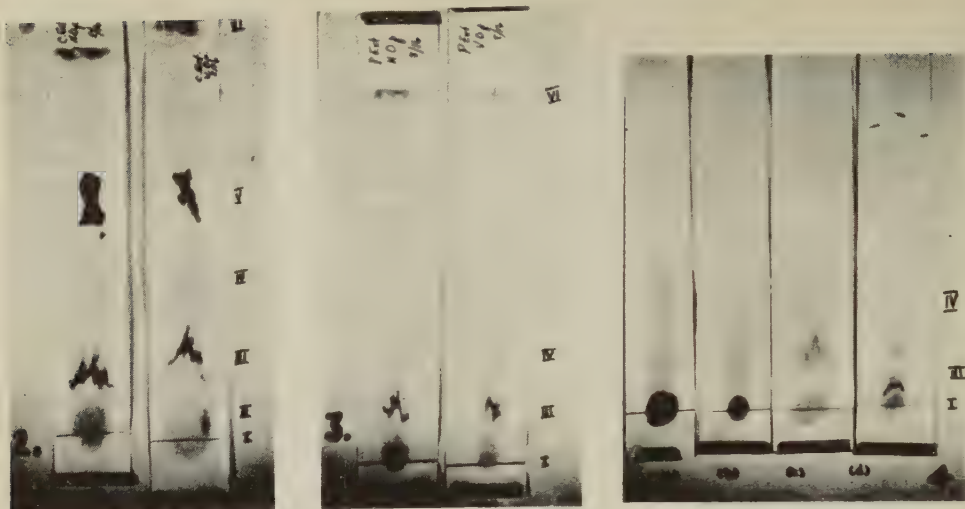


Figure 2. Electrophoretic-chromatogram of crude extracts of *N* and *LTV Oscillatoria princeps*. (a) *N*, (b) *LTV*. Note the presence of Zone II in b which is not present in a. Otherwise, the over-all pattern is about the same. (See text for explanation).

Figure 3. Electrophoretic-chromatograms of purified enzyme mixtures from (a) *N* and (b) *LTV Oscillatoria*. These chromatograms are identical.

Figure 4. Electrophoretic-chromatograms of enzymes from *N* and *LTV Oscillatoria*. (a) Branching enzyme from *N*; (b) Branching enzyme from *LTV*; (c) Phosphorylase from *N*; (d) Phosphorylase from *LTV*. (See text for the methods).

Pure preparations of the enzymes from *N* and *LTV* extracts were obtained as described by fractionation and reprecipitation from 0.1 saturated ammonium sulfate. The electrophoretic patterns of each of these enzymes are shown in Figure 4. In all cases the patterns are remarkably identical.

Activity studies showed that of the five and six zones defined in the

Table 1. Rate of Migration of Zones in Extracts from Normal and Variant Cultures of *Oscillatoria princeps* Under the Influence of 4 v./cm. and 4 m.a. for 12 hours at pH 6.8.¹

Zone	Distance Migrated (Total cm.)		Rate of Migration (cm./hr.)	
	Normal	Variant	Normal	Variant
I	—	—	—	—
II	—	0.5	—	0.04
III	3.2	4.0	0.26	0.33
IV	7.3	7.6	0.60	0.63
V	11.0	11.2	0.90	0.93
VI	17.1	18.2	1.42	1.51

¹ Averages of 12 determinations.

Table 2. *Phosphorylating Enzyme Activity of the Various Zones Separated by Electrophoresis of Crude, Purified Extracts, and Purified Enzyme Preparations.*

Fraction	Activity	Zones ¹		Rate of Migration (cm./hr.)	
		Normal	Variant	Normal	Variant
CRUDE EXTRACT	P-lase	III	III	0.26	0.33
	B.E.	IV	IV	0.60	0.63
PURIFIED EXTR. (0.8 sat. ammonium sulfate fractions)	P-lase	III	III	0.25	0.23
	B.E.	IV	IV	0.69	0.68
PURIFIED ENZYME (reprecipitation 0.1 sat. ammon. sulfate)...	P-lase	III	III	0.22	0.23
	B.E.	IV	IV	0.59	0.62

¹ Zone numbers correspond to the locations shown in Figure 2. P-lase is *phosphorylase*; B. E. is *branching enzyme*.

patterns of N and LTV strains in the crude extract, only two zones (Zones III and IV) exhibited phosphorylating and branching action. Zone III showed exclusively phosphorylase action, a blue iodine-staining polyglucoside being formed from buffered glucose-1-phosphate substrates. Zone IV exhibited branching enzyme activity. A branched sugar, which gave a reddish-violet color with iodine was formed by the fraction eluted from this zone. Table 2 shows a summary of the results of activity studies in the two zones mentioned in the crude extract, purified mixture, and pure enzymes preparations.

Measurement of the optical density of the eluted zones after staining with Naphthalene Black showed that, in the case of Zones III, no detectable differences were observed between N and LTV extracts. Hence, it appears that the concentration of phosphorylase is approximately the same in both strains of this alga.

Zone IV of N strains exhibited a greater concentration of the dye than Zone IV of LTV strains. The differences observed in optical density of the resulting elutions were significant. It would appear from these studies, that branching enzyme is present in greater concentration in N strains of *Oscillatoria princeps* than in LTV strains of this alga. However, it must be borne in mind that the crudeness of the method allows only a limited interpretation of the results from these studies. The results are by no means conclusive, only indicative.

Discussion

The physical constants (Michaelis-Menten, pH optima, etc.) of the enzymes involved in polyglucoside synthesis in N and LTV strains of *Oscillatoria princeps*, have been shown to be identical (Fredrick 1954). The che-

mical action of the branching enzymes derived from N and LTV strains of this alga, has also been shown to be the same (Fredrick and Mulligan 1955). No detectable structural differences or differences in mode of action have been observed which would serve to explain the synthesis of a less-branched polyglucoside by LTV strains of this plant.

Electrophoretic studies emphasized the similarity of the enzymes in both strains. As can be seen from Figures 2—4, and from Table 2, the enzymes, their rates of migration, etc. are remarkably identical in both N and LTV strains. The only difference apparent in the chromatograms (cf. Figure 2) is the presence of a slow-moving protein in the LTV extract which is not present in the N extract. However, it was subsequently proven that this zone had no influence on the actual polyglucoside synthesized by this alga. So, that while the presence of this zone in LTV chromatograms does serve as a distinguishing feature of LTV strains, it has no bearing on the immediate problem of the polyglucoside synthesized by LTV strains of *Oscillatoria princeps*.

It had been hypothesized, that the synthesis of a less-branched polyglucoside by LTV strains was due to a decrease in absolute concentration of branching enzyme in these strains of the alga. No clear-cut proof of this hypothesis has yet been presented. Cases are known in other organisms where a decrease in the activity of an enzyme or a group of enzymes has been observed as a mechanism of biochemical evolution giving rise to mutants from wild-type organisms. In yeast, for example, Ephrussi and Hottinguer have shown that the inheritance of a cytoplasm character is connected with a loss of respiratory enzymes (1951). The difference in the cellulolytic power (a complex enzyme function) in *Collybia velutipes* between wild-type and mutant strains, has been effectively demonstrated by Aschan and Norkrans (1953).

The studies of eluted, Naphthalene Black-dyed fractions of Zones IV in N and LTV chromatograms, indicated that a difference in concentration of branching enzyme in these two strains of this blue-green alga was present. These results were only indicative of such a difference. Further work is contemplated along these lines, however.

Assuming that there is indeed, a difference in the concentration of branching enzyme in these two strains, then the validity of the original hypothesis (Fredrick 1952, 1953) could be established. The loss of branching enzyme by the LTV strain explains the difference in the type of sugar synthesized by this strain as compared to N *Oscillatoria princeps*. Hence, the morphological differences (Fredrick 1953), the chemical differences reported (Fred-

rick 1953 b), and the enzymatic differences, can be resolved with a genetic difference. It is possible that this may be a path-way for the evolution of new species in this blue-green alga.

Conclusions

Electrophoretic-chromatographic patterns of enzyme extracts of N and LTV *Oscillatoria princeps* show a remarkable similarity. The only difference observed was the presence of a slow-moving protein fraction in the LTV strain which was absent from the N strain. This fraction proved to have no function in the synthesis of polyglucosides by this alga.

The rates of migration of the phosphorylases and the branching enzymes of these two strains were identical. No differences were apparent in the chromatographic patterns.

A greater concentration of branching enzyme in N strains was noted. This indicated that a possible explanation was feasible for the differences in polyglucosides synthesized by these two strains.

The hypothesis is offered that biochemical evolution proceeds in this alga by a progressive decrease in the absolute concentration of branching enzyme.

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References

- Aschan, K. & Norkrans, B.: A study in the cellulolytic variation for wild-types and mutants of *Collybia velutipes*. — *Physiol. Plant.* 6: 564. 1953.
- Buzzati-Traverso, A.: Paper chromatographic patterns of genetically different tissues: A contribution to the biochemical study of individuality. — *Proc. Natl. Acad. Sc.* 39: 376. 1953.
- Ephrussi, B. & Hottinguer, H.: Cytoplasmic constituents of heredity on an unstable state in yeast. — *Cold Spring Harb. Symp.* 16: 75. 1951.
- Fredrick, J.: Preliminary studies on the synthesis of polysaccharides in the algae. — *Physiol. Plant.* 4: 621. 1951.
- A polysaccharide variant of *Oscillatoria princeps*. — *Physiol. Plant.* 5: 37. 1952.
- Induction of polysaccharide variants in *Oscillatoria princeps* by low temperatures. — *Physiol. Plant.* 6: 96. 1953.
- Branching characteristics of *Oscillatoria polyglucosides*. — *Physiol. Plant.* 6: 100. 1953.
- Kinetics of polysaccharide formation in extracts of *Oscillatoria princeps*. — *Physiol. Plant.* 7: 182. 1954.
- Proposed molecular structure for straight and branched polymers of glucose. — *Physiol. Plant.* 8: 288. 1955.

- & Mulligan, F.: Mechanism of action of branching enzyme from *Oscillatoria* and the structure of branched dextrins. — *Physiol. Plant.* 8: 74. 1955.
- Hadorn, E. & Mitchell, H.: Properties of mutants of *Drosophila melanogaster* and changes during development as revealed by paper chromatography. — *Proc. Natl. Acad. Sc.* 37: 650. 1951.
- Haugaard, G. & Kroner, T.: Partition chromatography of amino acids with applied voltage. — *J. Amer. Chem. Soc.* 70: 2135. 1948.
- Reid, W.: Estimation and separation of the pectinesterase and polygalacturonase of micro-fungi. — *Nature* 166: 569. 1950.

The Interaction of Photosynthesis and Respiration and Its Importance for the Determination of ^{14}C -Discrimination in Photosynthesis

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1 Introduction

Van Norman and Brown in 1952 published the results of some admirable experiments with the green alga *Chlorella* according to which $^{13}\text{CO}_2$ is assimilated at a rate 2 per cent lower than $^{12}\text{CO}_2$, but $^{14}\text{CO}_2$ at a rate 14 per cent lower than $^{12}\text{CO}_2$. The statement about $^{13}\text{CO}_2$ was in perfect agreement both with previous investigations (3 per cent according to Nier and Gulbransen 1939) and with what is theoretically to be expected. The statement about $^{14}\text{CO}_2$ agrees well with the only previous measurements of $^{14}\text{CO}_2$ discrimination by Weigl and coworkers 1949, 1951, although these measurements were criticized by van Norman and Brown. Theoretically, however, the assimilation rate of $^{14}\text{CO}_2$, as compared with the rates of $^{12}\text{CO}_2$ and $^{13}\text{CO}_2$, seems rather astonishing. A discrimination of $^{14}\text{CO}_2$ against $^{12}\text{CO}_2$ of about 4 per cent, the double value of that for $^{13}\text{CO}_2$, would have seemed more likely.

If the discrimination mentioned is to be measured it is necessary to know whether respiration and photosynthesis are going on independently of each other. In order to investigate this, experiments were made by van Norman and Brown using oxygen tracers and a recording mass-spectograph. No influence of light was found on the rate of respiration as measured by the uptake of $^{18}\text{O}_2$ from the outside medium. These results were considered a proof that respiration and photosynthesis are going on independently of

each other although the two authors mention that because O_2 -uptake and O_2 -release are independent in light this must not necessarily be the same for CO_2 .

The scientists working with radiocarbon dating have presented first class measurements of the specific activity of ^{14}C in recent wood. Unfortunately we have no reliable measurements of the specific activity of ^{14}C in the atmospheric air and presumably it is too late now to make such determinations because of the experiments with H-bombs.

In freshwater some few determinations of the ^{14}C -content in the submerged plants as compared with the content in the CO_2 -system of the water has been made by Deevey et al. 1954. They seem to indicate that that ^{14}C -discrimination in photosynthesis under natural static conditions in water is only slight.

In the present article the high discrimination apparently found in the experiments made by van Norman and Brown will be recomputed. It will further be shown that the photosynthetic and the respiratory mechanisms do not work independently of each other although the O_2 -metabolism apparently indicates this to be so.

When describing the ^{14}C -method for measuring organic productivity in the sea — Steemann Nielsen 1952 — it was already rendered probable that CO_2 -uptake by photosynthesis and CO_2 -release by respiration do not take independently of each other. It was therefore pointed out that the ^{14}C -method cannot be used for measuring the rate of photosynthesis at low light intensities where the rate of respiration is the same or lower as the rate of photosynthesis.

An example may be mentioned. In an experiment with *Chlorella pyrenoidosa* the same rate of photosynthesis (gross production) was found at 7,000 lux when using either the ^{14}C -method or an oxygen method (Winkler analyses) if the photosynthetic quotient was put at 1.2. At 300 lux, which was just below the compensation point, the ^{14}C -method, however, gave a value (gross production) which was only 31 per cent of the value obtained by using the oxygen method. If the rate of respiration is higher than the rate of photosynthesis, the intermixing of the two processes has thus a considerable influence on the measurements of photosynthesis with the ^{14}C technique.

At high light intensities a correction for the measurements of gross production by the ^{14}C -method was introduced corresponding with 40 per cent of the rate of respiration and thus about 4 per cent of the maximum rate of photosynthesis which normally was found to be about 10 times the rate of respiration. As will be shown below, the correction should rather be put at 6 per cent.

2. Experiments on the Release by Respiration of $^{14}\text{CO}_2$ Previously Assimilated

One of the easiest ways of investigating the intermixing of CO_2 -release due to respiration and CO_2 -uptake due to photosynthesis seems to be by measuring in the light and in the dark the release of ^{14}C previously assimilated. If according to van Norman and Brown's measurements with oxygen tracers it is a priori assumed that the rate of respiration is the same in the light and in the dark, any decrease in the release of ^{14}C must be due to some interaction either between CO_2 -uptake and CO_2 -release or between respiration and photosynthesis. The real reason for such an interaction may, however, be searched for in several instances, such as:

1. A preferable assimilation inside the cells of CO_2 produced by respiration. CO_2 from the outside is to diffuse into the cells before being assimilated by the photosynthetic apparatus, whereas such a diffusing is not necessary for respiratory CO_2 produced inside the cell.

2. A preferable use for respiration of organic matter just produced by photosynthesis.

3. An intermixing of respiration and photosynthesis due to joint pools of intermediates in the two processes.

4. O_2 -consumption as measured according to the uptake of tracer oxygen in light is not due exclusively to oxidation of hydrogen produced during the decomposition of organic matter but also in an appreciable extent to oxidation of hydrogen produced from water during the photochemical partial processes in photosynthesis.

5. The mechanism of respiration differs in the light and in the dark.

6. The carboxylation in photosynthesis may demand HCO_3^- which also may be released in respiration instead of CO_2 . In spite of carbonic anhydrase the process $\text{CO}_2 + \text{H}_2\text{O} \rightleftharpoons \text{H}_2\text{CO}_3$ is not momentary. A preferable uptake of $\text{CO}_2(\text{HCO}_3^-)$ produced by respiration could therefore be expected.

Of these 6 possibilities the first will in the present article be shown not to be the normal cause of the intermixing of CO_2 -release and CO_2 -uptake in light. According to work done by Calvin's group in Berkeley (cp. e.g. Calvin and Massini 1952) it seems further unlikely that organic matter just produced by photosynthesis is preferably used for respiration or that joint pools of intermediates are of decisive importance in this respect.

One of the main experiments will now be described. Cells from a 3 days old fast-growing culture of *Chlorella pyrenoidosa* (Warburg strain) were transferred to a medium, pH 8.2, containing a total amount of $^{14}\text{CO}_2$ in the medium corresponding to 7.5×10^6 counts per minute. After 2 hours at 8,000 lux 20°C ., during which period about 25 per cent of the ^{14}C was assimilated, the algae were centrifuged in the dark and washed twice with

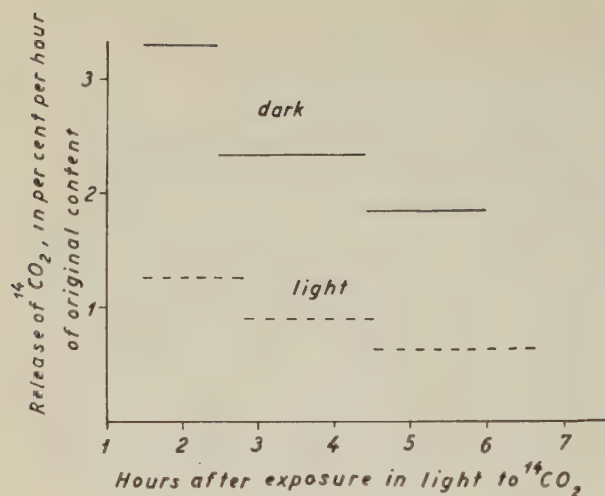


Figure 1. The release of previously assimilated $^{14}\text{CO}_2$ in the light and in the dark. *Chlorella*.

a bicarbonate buffer containing 0.03 per cent free CO_2 . The algae were then transferred to a fresh medium and kept in the dark at 20°C . for $1\frac{1}{2}$ hours before the experiments proper were started.

One of the reasons why these experiments were not started at once was that $^{14}\text{CO}_2$ found in the cells due to previous dark fixation during the first hour in the dark to a high degree interchanged with the normal CO_2 in the outside medium, as no $^{14}\text{CO}_2$ was present in the outside medium. As the loss of tracer due to this process at the beginning may be as high or even higher than the loss due to respiration, some time must elapse before the influence of light on the $^{14}\text{CO}_2$ produced by respiration can be investigated.

Another reason for not starting the experiments immediately after the uptake of tracer carbon is finished, is that the rate of respiration in the dark for some time after a change in pH may be rather different from the normal one. Such a change is necessary in some special experiments. In light, on the other hand, transitory differences in the metabolism of *Chlorella* due to a shift in pH seem to be of very short duration only (Steemann Nielsen 1955). This complication can of course be totally avoided by making the whole experiment at the same pH. As this, however, was not the case in the preliminary experiment concerning the influence of light on the release of previously assimilated ^{14}C published by Steemann Nielsen 1953, no stress can be laid on the absolute value given there, although the result in principle agrees very well with the results of later experiments.

After the $1\frac{1}{2}$ hours mentioned the *Chlorella* suspension was centrifuged, washed, and centrifuged again. The cells were then suspended in 100 ml of the medium and distributed to 2 bottles with glass stoppers each containing

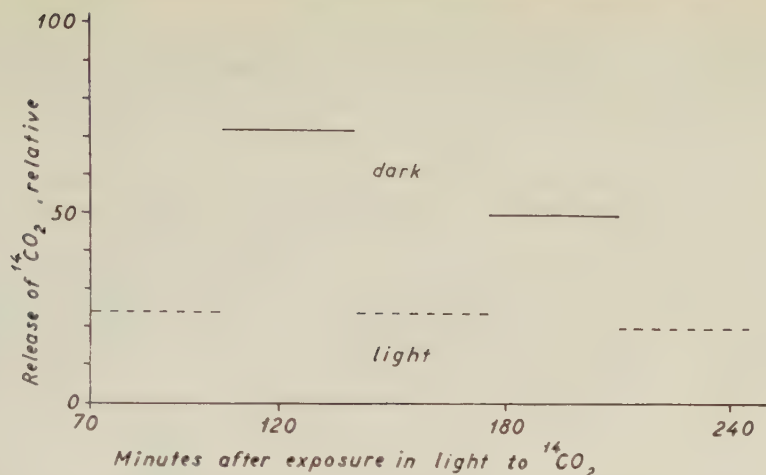


Figure 2. The release of previously assimilated $^{14}\text{CO}_2$ in the light and in the dark. *Myriophyllum*.

50 ml, one of which was put in the dark and one in the light of about 8,000 lux.

After about 1 hour, 3 and 5 hours, 10 ml of each bottle were filtered through a millipore filter. The activity of the $^{14}\text{CO}_2$ in the filtrate was determined according to Steemann Nielsen 1952: the CO_2 was precipitated as BaCO_3 .

In Figure 1 the $^{14}\text{CO}_2$ -release in the light and in the dark is given during the times indicated as per cent released by hour of the amount found in the algae at the start of the proper experiments. In light from 3.3 to 1.8 per cent per hour was released, in dark from 1.3 to 0.6 per cent. It is thus seen that tracer-carbon during the whole experiment was given off in the light at a rate of 33–40 per cent of the rate in the dark. During the five hours the release of $^{14}\text{CO}_2$, both in the light and in the dark, decreased by about 50 per cent.

In order to investigate whether the different behaviour of the $^{14}\text{CO}_2$ production in light and dark should be caused by a preferable assimilation in photosynthesis of interior CO_2 produced by respiration, some similar experiments lasting for about 1 1/2 hours were made at a concentration of free CO_2 150 times as high as in the experiment presented in Figure 1 (4.5 per cent). The light intensity was so low (500 lux) that the rate of photosynthesis just equalled the rate of respiration. A similar difference between the release of $^{14}\text{CO}_2$ in light and dark was, however, found. In one experiment about 50 per cent of the $^{14}\text{CO}_2$ given off in the dark was given off in the light in another at about 30 per cent, in a third at about 50 per cent. No preferable

uptake in respiration of interiorly produced oxygen was found in the light by van Norman and Brown. The idea of preferable uptake of respiratory CO_2 due to diffusion may thus be rejected as being the main cause of the different rate of $^{14}\text{CO}_2$ released in the light and in the dark.

Other plant species seem to behave like *Chlorella*. In Figure 2 is thus shown a series of experiments at 20°C . with a submerged specimen of the phanero-gamic aquatic *Myriophyllum amphibium* from a river on Cape Cod, Mass., U.S.A. After photosynthesis in a medium containing $^{14}\text{CO}_2$ for 2 hours, during which time 25 per cent of the tracer was assimilated, the plant was washed in the dark in water without $^{14}\text{CO}_2$ for 70 minutes. It was subsequently placed alternately in the light (8,000 lux) and in the dark for 35 minute periods. The concentration of free CO_2 was 0.6 per cent, pH about 5. No ^{14}C was originally found in the carbon-dioxide system of the water.

The plant specimen was fastened to a perforated celluloid plate inside a closed cuvette, which was placed on a rotating disc in a water bath. As the plate was shorter than the cuvette, an effective stirring was produced inside the cuvette (about details cp. Steemann Nielsen 1942). The release of $^{14}\text{CO}_2$ during the experiment was measured in the same way as in the *Chlorella* experiments. $^{14}\text{CO}_2$ was given off in the light at a rate of about 40 per cent of the rate in the dark.

If all experiments are taken as a whole, it can be stated that in light only from 30—50 per cent of the labelled CO_2 given off due to the respiration in the dark is released in the light. An average of 60 per cent of the respiratory CO_2 is thus in light identical with CO_2 taking part in photosynthesis. The background of this behaviour is most likely to be found in one of the three possibilities stated as Nos. 4—6 on p. 947 or there may be still another reason.

If the rate of respiration is 10 per cent of the rate of photosynthesis which is normally found at a high light intensity, the rate of photosynthesis (real assimilation) as measured through the assimilation of the tracer carbon is

$$100 - \frac{10 \times 60}{100} = 94 \text{ per cent of the real value. A correction must therefore be}$$

made if the ^{14}C -technique is used for measuring gross production. The correction 6 (6.4) per cent (4 per cent according to Steemann Nielsen 1952) is of course only correct if the rate of respiration is 10 per cent of the rate of photosynthesis at the high light intensity at which the measurements are made. If the rate of respiration is 20 per cent the correction for the measurements made with the ^{14}C technique should be 14 per cent. When the standard correction 6 per cent is used in this case the computed photosynthesis rate is 8 per cent too low. If the rate of respiration is $\frac{1}{4}$ of the rate of photosynthesis at a high light intensity (an extreme quotient which in plankton algae in

nature only on rare occasions will be approached, see Steemann Nielsen and Al Kholy in press) the measurements by the ^{14}C -technique corrected in the standard way are 13 per cent too low.

3. ^{14}C -Discrimination in Photosynthesis

As mentioned in the introduction, $^{14}\text{CO}_2$ according to van Norman and Brown 1952 is assimilated during photosynthesis at a rate 14 per cent lower than $^{12}\text{CO}_2$, whereas $^{13}\text{CO}_2$ is only assimilated at a rate 2 per cent lower. The measurements were made with the use of a recording mass spectrograph. They are of a technically very high standard.

It must, however, be mentioned that in these experiments the discrimination of ^{13}C was measured under static conditions, the ^{14}C -discrimination under kinetic conditions. $^{14}\text{CO}_2$ was first introduced into the *Chlorella* culture at the start of the experiment, whereas no enrichment was done of $^{13}\text{CO}_2$ relative to $^{12}\text{CO}_2$. In measuring ^{13}C -discrimination it is therefore neither necessary to take any dark uptake into account nor to consider any possible interaction between photosynthesis and respiration. Both circumstances have to be considered when dealing with $^{14}\text{CO}_2$.

Let us first consider the dark uptake of $^{14}\text{CO}_2$. Van Norman and Brown supposed that the uptake of $^{14}\text{CO}_2$ due to isotope interchange was the same during the first 35 minutes in light as during the previous 35 minutes in the dark and they made their correction according to this supposition. This is not, however, correct.

Figure 3 shows a typical curve illustrating the dependence of dark interchange of $^{14}\text{CO}_2$ on time. During the first 35 minutes the rate is at least twice the rate during the subsequent 35 minutes. For some reason or other the dark interchange of $^{14}\text{CO}_2$ was extremely high in van Norman and Brown's experiment — about 5 times as high as normally found in a first class culture of *Chlorella*. It is therefore of real importance for the determination of the isotope discrimination if the correction made is twice the correction really to be done. If the authors had used the correct correction, ^{14}C -discrimination would in this way have been 10 instead of 14 per cent. A value of 10 per cent must, however, still be considered too high.

The interaction of photosynthesis and respiration shown in section 2 is the other important factor to be considered if a recalculation of the ^{14}C -discrimination in photosynthesis on the basis of van Norman and Brown's data has to be made. The rate of respiration was about 8 per cent of the rate of photosynthesis (total assimilation) according to Fig. 3 in the article mentioned. If it is assumed that 60 per cent of the respiratory CO_2 is identical with CO_2

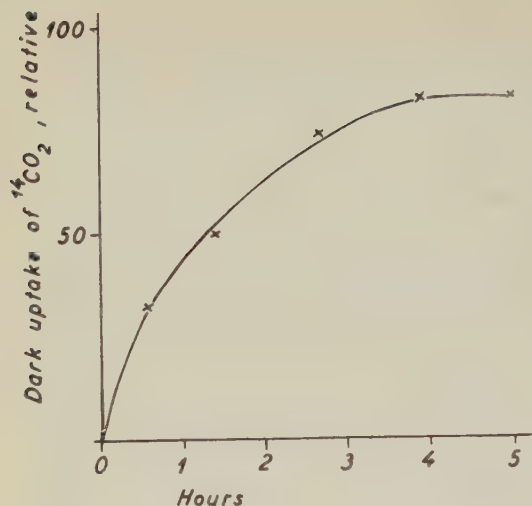


Figure 3. Dark uptake of $^{14}\text{CO}_2$ in *Chlorella*.

assimilated during photosynthesis this means that only $100 - \frac{8 \times 60}{100} = 95$ per cent of $^{14}\text{CO}_2$ is assimilated. In stead of being 10 per cent the ^{14}C -discrimination in photosynthesis is thus only about 5 per cent. This value is theoretically in perfect agreement with the value for ^{13}C as measured by van Norman and Brown, and by Nier and Gulbransen. It is also in good agreement with Deevey's measurements regarding specific activity of ^{14}C in the CO_2 of lake water and in the submerged plants from the same locality.

Summary

Although oxygen metabolism shows no interaction of photosynthesis and respiration, CO_2 metabolism shows such an interaction amounting to about 50—70 per cent of the respiratory rate.

It is shown that the main cause of the interaction is not found in a preferable assimilation of respiratory CO_2 due to the shorter distance for diffusion as compared with CO_2 from the outside medium. Instead some other possible explanations of the fact are presented.

The ^{14}C -discrimination in photosynthesis is recalculated on the basis of the measurements by van Norman and Brown 1952. Besides taking the interaction of photosynthesis and respiration into account, it is at the same time necessary to recalculate the influence of isotopic interchange independent of photosynthesis. The authors mentioned supposed the dark assimilation of

$^{14}\text{CO}_2$ to be constant during the first 70 minutes. This is not, however, correct. Taking all evidence into account the discrimination of ^{14}C as compared with ^{12}C is about 5 per cent.

References

- Calvin, M. and Massini, P.: The Path of Carbon in Photosynthesis (XX. The Steady State). — *Experientia* 8: 445. 1952.
- Deevey, E. S., Gross, M. S., Hutchinson, G. E. and Kraybill, H. L.: The Natural C^{14} Contents of Materials from Hard-Water Lakes. — *Proceed. Nat. Acad. Science.* 40: 285. 1954.
- Nier, A. O. and Gulbransen, E. A.: Variations in the Relative Abundance of the Carbon Isotopes. — *J. Amer. Chem. Soc.* 61: 697. 1939.
- van Norman, R. W. and Brown, A. H.: The Relative Rates of Photosynthetic Assimilation of Isotopic Forms of Carbon Dioxide. — *Plant. Physiol.* 27: 691. 1952.
- Steemann Nielsen, E.: Der Mechanismus der Photosynthese. — *Versuche mit Fucus serratus und anderen Submersen.* — *Dansk Bot. Ark.* 11, nr. 2: 1. 1942.
- The Use of Radio-active Carbon (C^{14}) for measuring Organic Production in the Sea. — *Journ. du Conseil Intern. l'Explor. de la Mer.* 18: 117. 1952.
- Carbon Dioxide Concentration, Respiration during Photosynthesis and Maximum Yield of Photosynthesis. — *Physiol. Plant.* 6: 316. 1953.
- Carbon Dioxide as Carbon Source and Narcotic in Photosynthesis and Growth of *Chlorella pyrenoidosa*. — *Ibidem* 8: 317. 1955.
- and Al Kholy, A. A.: The Use of the ^{14}C -Technique in Measuring Photosynthesis under Condition of Deficiency in P or N. — *In press.*
- Weigl, J. W. and Calvin, M.: An Isotope Effect in Photosynthesis. — *Journ. Chem. Phys.* 17: 210. 1949.
- Warrington, P. M. and Calvin, M.: The Relation of photosynthesis to Respiration. — *J. Amer. Chem. Soc.* 73: 5058. 1951.

A New Method for the Determination of the Turgor of Plant Tissues

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Introduction

A large number of studies on the permeability of the protoplasm of plant cells and tissues has been made with plasmolytic methods of different kinds. Most of the methods include microscopic investigations, especially when it is a question of estimating the changes in the shape of the protoplast and factors connected with it. Such determinations involve a great deal of labour as many cells must be studied separately in order to obtain statistically reliable values. In determinations of these kinds changes often occur in the concentration of the plasmolyticum during the study under the microscope. Further, for microscopic investigations very thin slices of the material must be used. It has been shown by many authors that the permeability is strongly influenced by cutting and other acts of interference on the living tissue, (cf. Lepeschkin, 1938). Thus, in such cases one always runs the risk of getting wound effects and different permeability values as compared to those of cells in natural surroundings.

With the method which will be described here, most of the difficulties, here mentioned, are greatly reduced as, on the one hand, microscopic investigation is not required; on the other hand, considerably larger amounts of tissue can be employed without changing the accuracy of the determination.

Principle of the Method

A rod of any kind of solid material which is fastened at one end and made to vibrate will keep on vibrating for a short time with a constant frequency

which is characteristic for the rod in question, for instance a tuning fork. If the rod is straight without any obvious geometric irregularities, the vibrations of fundamental frequency are predominant. But even vibrations of higher harmonics can be found. The vibrations of the first, second and higher harmonics are especially evident if the vibrating body is geometrically irregular. These frequencies with which the body can vibrate on its own are usually called its resonance frequencies. As long as the physical conditions as rigidity and mass of the body are unaltered, its resonance frequencies are constant. Any change in the mechanical properties will, however, cause an alteration in these frequencies.

The body can be forced to vibrate with other frequencies by applying an external oscillating force. Considerably more energy has to be administered, however, in order to maintain the vibrations at a certain amplitude as compared to that which is required when the body vibrates with its own resonance frequencies. This can be illustrated with a piece of steel wire fastened at one end and placed in front of an electromagnet. If the magnet is fed with alternating current of varying frequency from an oscillator, the wire will vibrate violently only when the A.C. frequency fed to the electromagnet is the same as the resonance frequency of the wire. A.C. frequencies even very close to the resonance frequency of the steel wire will have only a very small effect — if the damping of the wire is not too great. By this method it is thus possible to determine the resonance frequencies of solid bodies (cf. Barkhausen, 1940).

The same principle can now be applied for the determination of the rigidity of plant tissues and can be used for the estimation of turgor and factors connected with water relations of the cell. If the tissue under investigation is provided with a little piece of thin steel at the free end, it will behave in front of an electromagnet as the steel wire mentioned above. As the rigidity of the plant tissue and, to a much smaller extent, even the mass in contradiction to that of the steel wire is very easily altered by changing, for instance, the water content, these changes can be measured by determining the resonance frequency. A schematic outline of the arrangement for such determination can be seen in Figure 1.

As the living tissue has a complicated geometry, frequencies of higher harmonics often occur. In one determination it is therefore necessary to make sure that one is always studying the changes of one and the same frequency when altering the external conditions. If the different resonance frequencies are close to each other it is, namely, easy to make the mistake of »jumping» over to another harmonics. Before measurements are performed it is advisable to investigate a wide range of frequencies to find out where the different resonances are located. The resonance frequencies depend

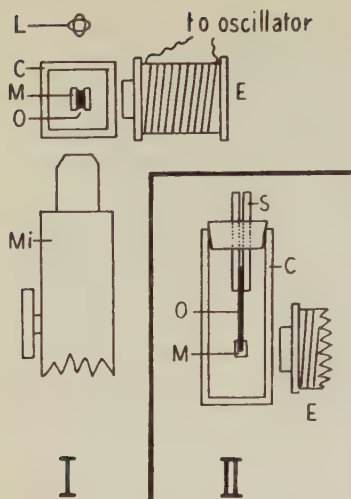


Figure 1. *Schematic view of the apparatus used for turgor measurements.* I. The arrangement seen from above. II. From the side. The plant tissue (O) to be investigated is fastened in a glass tube (S) and placed in a glass cuvet (C) with stopper. Around the tip of the tissue a piece of thin de-tempered steel (M) is bent. At one side of the cuvet the pole of an electromagnet (E) is placed facing the steel plate on the tissue. The tissue can be observed through a low power horizontal microscope (Mi). In order to facilitate observations, an electric lamp (L) is placed behind the cuvet. The electromagnet used for the experiments described in this paper had a resistance of 600 ohms. Electromagnets from common ring bells can be used, but more powerful ones work better. The alternating current of varying periodicity was supplied from a laboratory oscillator of common type.

on, among other factors, the shape, length and thickness of the investigated material. The author has found that the best results will be obtained if the main frequency lies around 70—80 cycles per second. For a piece of potato tuber parenchyma, 1×1 mm in cross section and fully turgescant, this will mean a length of about 10 mm.

For the fastening of the piece to be investigated, it is difficult to give any definite advice as the material is so variable. It may be mentioned for a guidance that roots and thick homogeneous material as pieces of potato tuber parenchyma and similar material can easily be fastened by pushing the tissue up in glass tubes of corresponding inner diameters. Leaves and thin material are best fastened in between two glass slides. In the choice of magnetic material for application at the plant tissue, very thin steel, 0.025 mm in thickness, as used in the automobile industry for measuring purposes, is suitable. Before use, however, it should be de-tempered. It will then become very pliable. The size of the piece required varies, but for tissues mentioned in the foregoing pieces 1×2 mm were appropriate.

Some Applications of the Method

1. *Determination of osmotic value of the cell sap.* — Three examples of the possibilities of determining of the osmotic value of the cell sap will be given here.

A tissue, immersed in a hypertonic solution, will gradually lose its water until equilibrium is reached. In connection with this water loss, the turgor

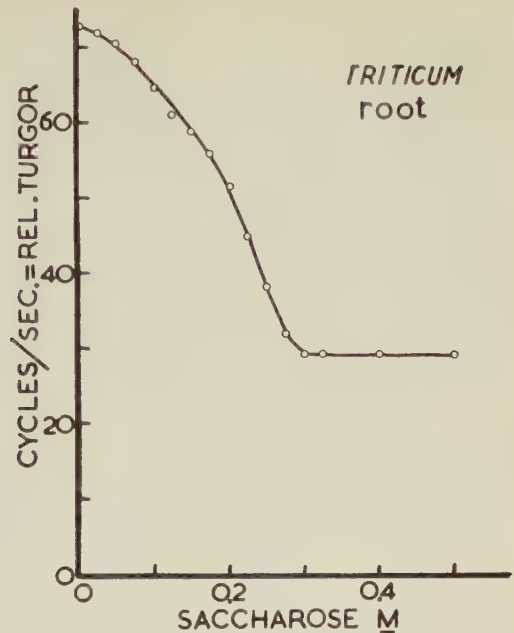


Figure 2. Change in the turgor of a wheat root, when immersed in saccharose solutions of varying concentrations. Length of the root: 10 mm.

and wall pressure will decrease. As this happens the rigidity of the whole tissue decreases. This decrease will continue as long as the protoplast is pressed against the cell wall membrane. When incipient plasmolysis occurs further water loss will have no effect on the rigidity. Only if considerable amounts of water are sucked out from the micellar system of the cellulose membrane, changes in the rigidity may occur even after plasmolysis.

The changes in the rigidity of a wheat root when immersed in solutions of saccharose of varying concentrations are seen in Figure 2. Before the resonance frequency was measured, the tissue was left in the solution for ten minutes, which is time enough for the tissue to come into equilibrium with the solution when changes in concentrations are as small as in this case (0.025 M). From the Figure it can be seen that as the concentration of the surrounding medium increases, the rigidity of the root decreases until a certain value for the concentration is reached, in this case between 0.275 M and 0.300 M. At higher concentrations no or very slight decrease can be seen. As the different cell layers in a wheat root differ in osmotic value, one can assume that the value which determines the place for the sudden bend of the curve into a straight line is that of the cell layer having the highest osmotic value for the cell sap. The fact that this change occurs so suddenly, as seen in the Figure, when the concentration of the solution is increased, suggests that there is only a small spread in osmotic values within the layer.

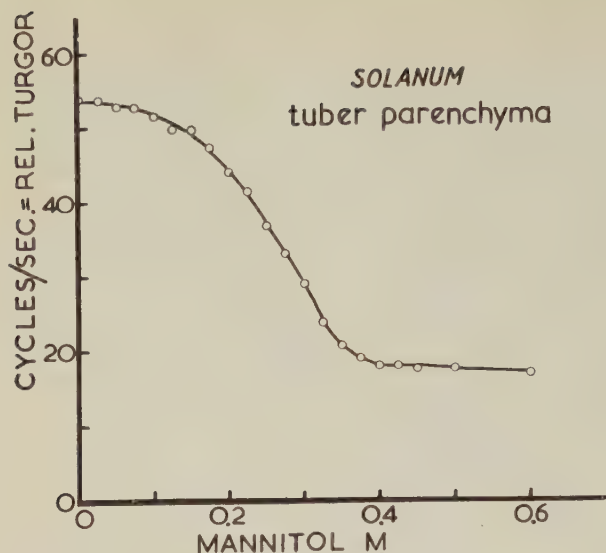


Figure 3. Change in the turgor of a piece of potato tuber parenchyma, when immersed in mannitol solutions of varying concentrations. Size of the piece of tissue: $12 \times 1 \times 1$ mm.

If the same measurements are performed on a small piece of parenchyma from potato tubers a curve will be obtained, which is shown in Figure 3. Principally the same shape of the curve is obtained except for a softer bending of the curve. It can be assumed that such a small piece of tissue as is used for these experiments is quite homogeneous and we do not have to count with different cell layers with distinct differences as to permeability and diffusion pressure deficit. The soft bending of the curve probably depends only on a certain spread of the osmotic values of the single cells. This is quite probable as the equilibrium starch — sugar in potato tuber cells can be easily shifted.

The method of determining the osmotic value by this method can be applied even to leaves or pieces of leaves. In Figure 4 are shown the values obtained with a small leaf of *Helodea densa*. In this case a small leaf was taken and the very tip thereof was covered with a little piece of steel (1×1 mm). Small pieces of a single leaf can also be investigated. This is especially important as the physiologic anatomy, for instance the osmotic value, may vary from one part to another (Meindl, 1934; Moder, 1922), causing a spread of the values obtained.

2. *Determination of cell permeability.* — In the previous section it was shown how the changes in turgor could be measured after the concentration of the surrounding medium had been changed. It is, of course, also possible to measure the change in rigidity of the tissue with time when immersed in an osmotically active solution. From the moment the tissue is placed in

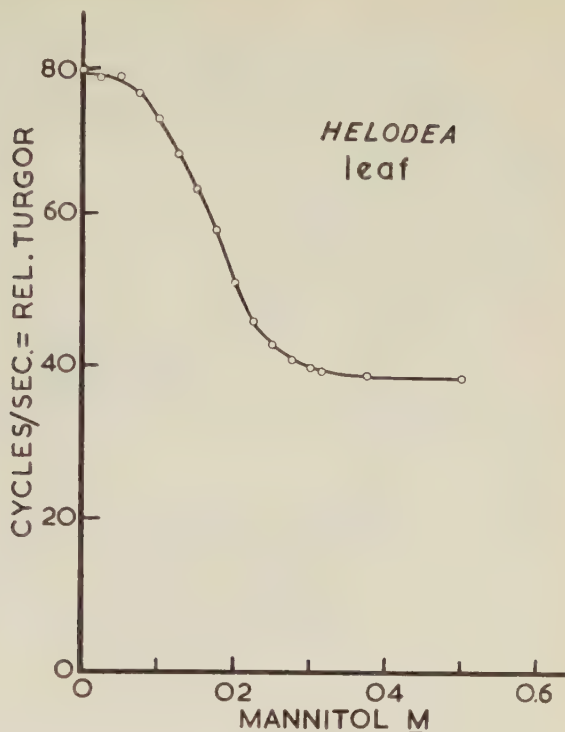


Figure 4. Change in the turgor of a leaf of *Helodea densa*, when immersed in mannitol solutions of varying concentrations.

the solution until osmotic equilibrium is obtained the rigidity of the tissue will gradually decrease. The speed of this decrease, i.e. the slope of the curve for the rigidity vs. time, will then be a measure of the permeability to the solvent provided the protoplasmic membranes are ideally semipermeable. As the permeability of the cellulose membrane to water and most soluble substances is assumed to be unlimited (exceptions, cf. Horié, 1954), any change in the slope of the curve will be a measure of the protoplasmic permeability to the solvent. Also the permeability to solutes can be determined in this manner. In Figure 5, curve No. 1, is shown the time course for the water movement from a thin slice of potato tuber parenchyma, which is immersed in 0.3 M solution of mannitol after having previously been kept in distilled water for 30 minutes. After about 30 minutes in the solution equilibrium is reached and the curve becomes parallel to the x-axis. If the mannitol solution is exchanged for distilled water, we get the time course represented by curve No. 2. The same exchange of solutions can be repeated several times and it is interesting to see the great reproducibility of the experiments. This makes it possible to determine even very small changes in the slope of the curves. It is obvious from the Figure that the first permeability curves differ from

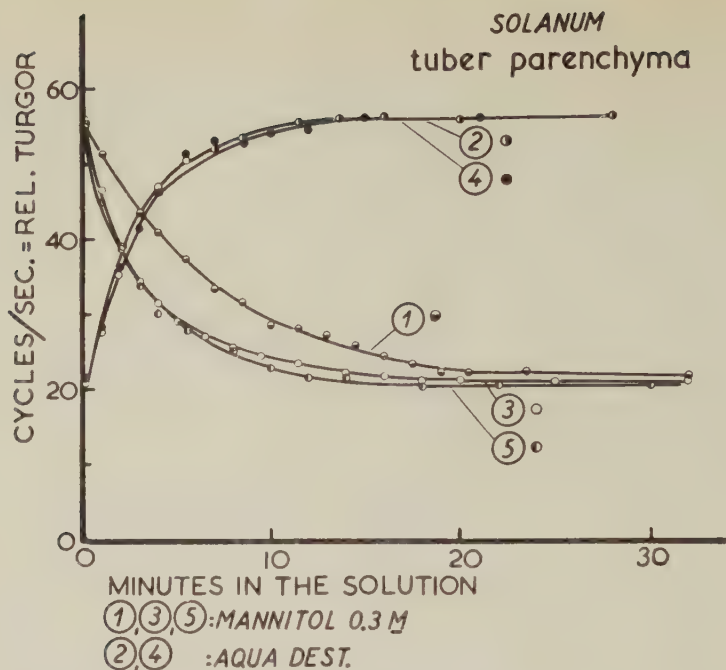


Figure 5. Time course of water movement out from and into a piece of potato tuber parenchyma when placed alternatingly in mannitol solutions and distilled water.

those obtained later on. The difference indicates that the permeability to water of the protoplasm is smaller in the first treatment with osmotically active substances as compared to the values obtained later when the same experiment is repeated. This finding is a good confirmation of the frequently expressed assumption that the outer protoplasmic boundary layer — the plasmalemma — is very much affected by the plasmolyticum which may also cause irreversible changes in the plasmatic system. This effect will be particularly evident if the concentration is high enough to cause plasmolysis.

The reproducibility of the determinations suggest that the method could be used for estimation of even comparatively small changes in the protoplasmic permeability. An example of such a determination is given in Figure 6. Here a piece of potato tuber parenchyma has been treated with mannitol and distilled water until a reproducible permeability curve has been obtained according to the experiments represented in Figure 5. The tissue has then been immersed in an osmotically isotonic solution which is $6.5 \times 10^{-6} M$ in respect to p-chlorophenoxyisobutyric acid (PCIB). This substance is known in these concentration to act as an antiauxin on the growth of

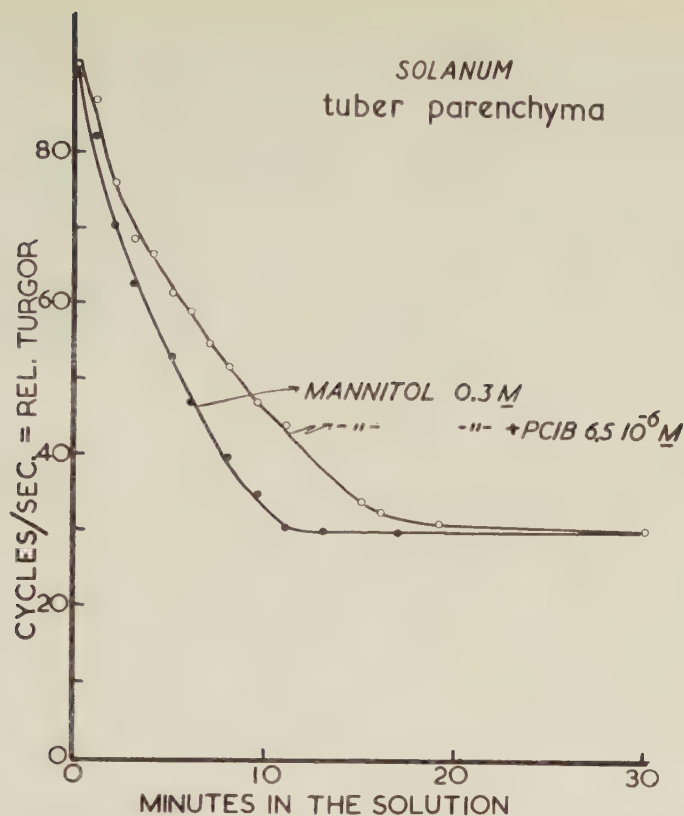


Figure 6. Time course of water movement out from a piece of potato tuber parenchyma, when placed in a) mannitol, 0.3 M; b) mannitol, 0.3 M + parachloroisobutyric acid, $6.5 \cdot 10^{-6} M$.

wheat roots (Burström, 1950). As mannitol is known as having no physiological effect on plant protoplasm (Burström, 1953) and does not permeate through the plasma membranes, any change in the slope of the curves must be due to a decrease in the permeability of the protoplasm to water. It is evident from the curves that one of the effects of PCIB consists in a lowering of the permeability to water of the protoplasm. That auxins and antiauxins have strong effects on the protoplasmic permeability to urea and other small molecules has been shown by several authors (cf. Masuda, 1955). This method of determination of the action of chemical substances opens up new possibilities for making rapid estimations. The fact that the same piece of material can be used for a long period of time suggests the possibility that several series of experiments can be performed without changing the experimental object.

The slope of the curve for the turgor changes, when a tissue is immersed in an osmotically active solution, depends on in addition to the permeability also the speed of the diffusion of the solution through the tissue. This is a factor which must be considered in a quantitative treatment of the curves as the thickness of the material often amounts to around one millimeter. The possibilities of determining the relationship between the slopes of the curves and the thickness of the material will be discussed in a future paper.

Summary

A method is described by means of which the turgor of plant tissues can be measured. The method can be used for determination of factors connected with water relations within the tissue as osmotic value and permeability.

The method is based upon the fact that a solid body which is caused to vibrate oscillates with a constant frequency characteristic for the body (resonance frequency). This frequency can be determined by determination of the frequency at which resonance phenomena occur. Any change in rigidity or mass of the body will change its resonance frequency.

A few examples of applications are given showing that equally or more accurate determinations are obtained with this method than with other methods.

The author is indebted to Dr. Vannevar Bush for suggesting the method and to Dr. Hellmuth Hertz for valuable discussions.

References

- Barkhausen, H.: Einführung in die Schwingungslehre. 2nd Ed. — Leipzig 1940.
- Burström, H.: Studies on growth and metabolism of roots. IV. Positive and negative auxin effects on cell elongation. — *Physiol. Plant.* 3: 277. 1950.
- Studies on growth and metabolism of roots. IX. Cell elongation and water absorption. — *Physiol. Plant.* 6: 262. 1953.
- Horić, K.: Permeability of the cell membrane of *Hydrodictyon reticulatum*. — *Cytologia* 19: 117. 1954.
- Lepeschkin, W. W.: Kolloidchemie des Protoplasmas. — Wissenschaftliche Forschungsberichte 47. Dresden 1938.
- Masuda, Y.: Der Einfluss des Heteroauxins auf die Plasmapermeabilität für Harnstoff und Alkylharnstoffe. — *Physiol. Plant.* 8: 527. 1955.
- Meindl, Tilde: Weitere Beiträge zur protoplasmatischen Anatomie des *Helodea*-Blattes. — *Protoplasma* 21: 362. 1934.
- Moder, Angela: Beiträge zur protoplasmatischen Anatomie des *Helodea*-Blattes. — *Protoplasma* 16: 1. 1932.

Action of Different Light Qualities on Simultaneous Photosynthesis and Nitrate Assimilation in Wheat Leaves

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(Received October 10, 1955)

Introduction

It can now be considered well established that light under certain circumstances is an indispensable factor for nitrate assimilation in plants, e.g. the reduction of nitrate and the linkage of the product to a suitable carbon compound, while this process in other cases can take place in complete darkness. Our knowledge about the mechanism of these reactions is in many essential aspects still very restricted, however. This is especially true of the light-dependent nitrate reduction. Warburg and Negelein (1920) thought that light acted through increasing the permeability for nitrate and the same opinion was held by Tottingham, Stephens, and Lease (1934). Lease and Tottingham (1935) assumed the light effect (at least the effect of shorter wave lengths) to be dependent on increased formation of chlorophyll and consequently better supply of carbohydrates, while Dittrich (1930) ascribed an important role to the increased transpiration and salt transport.

The simplest explanation would be that light via photosynthesis supplies the necessary carbohydrates for respiration and that during this latter process the needed quantities of energy and carbon compounds are liberated. Such a linkage of nitrate assimilation to respiration is normal in all plants or parts of plants that possess the ability to reduce nitrate in darkness. Experiments

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by Burström (1943) with detached wheat leaves nevertheless indicate that such an explanation cannot be correct. His results show on one hand that the light effect is more directly linked up with photosynthesis and on the other that it is not the supply of carbohydrates, necessary for respiration, that in itself acts as a limiting factor on nitrate assimilation. Leaves, that have a high content of sugars, are not able to reduce nitrate in darkness and even if they are placed in light, nitrate assimilation is very low if the carbon dioxide content of the surrounding air has a reduced value.

In recent years a more direct effect of light on nitrate reduction has repeatedly been proposed. Especially van Niels general formulation of photosynthesis:



has stimulated several investigators to assume analogous reactions for nitrate assimilation. Nitrate has then been considered as an alternative hydrogen acceptor to carbon dioxide (Rabinowitch 1945, van Niel, Allen and Wright 1953). The same lines of thought are the basis of another formulation of nitrate assimilation by Evans and Nason (1953). They were able to show that nitrate was reduced to nitrite when »grana» from leaves of soybean plants were irradiated in the presence of triphosphopyridine nucleotide (TPN) and nitrate reductase. According to them the first step in the light reaction is the formation of reduced triphosphopyridine nucleotide (TPNH) as proposed by Vishniac and Ochoa (1952), followed by the reduction of nitrate through the reduced coenzyme. The following system was believed to be working:



In the complete reduction of nitrate a more complicated scheme must be assumed. Thus it has been shown by Kessler (1955) that in green algae the reaction $\text{NO}_3^- \rightarrow \text{NO}_2^-$ is not affected at all by dinitrophenol (DNP), while the further reduction of nitrite is completely inhibited by this agent. Probably adenosinetriphosphate (ATP) is participating in the latter case and the possibility at once suggests itself that this compound can be generated in two ways, either through respiration in darkness or through photosynthetic phosphorylation (Arnon, Allen and Whatley 1954) in light. The light effect on nitrate reduction may thus be a rather complicated one.

The present paper does not deal with the problem from this purely biochemical point of view but from a more physiological one. The starting point

consists partly of Burströms demonstration of the linkage of nitrate reduction to photosynthesis and partly of the papers of Tottingham et al. (1928, 1934, 1935). These latter workers claim to have shown that the shorter wave lengths of light have a much stronger effect on nitrate assimilation than the longer ones. This statement seems to be inconsistent with Burströms results, at all events if one assumes the light to act only via photosynthesis. One way to solve this problem may be to determine simultaneously the action spectrum for both carbon dioxide and nitrate assimilation, and the present investigation is an attempt in this direction.

Methods

General remarks

The object of the investigation was to compare the carbon dioxide- and nitrate assimilation for a number of different wave length regions in the visible spectrum in order to find out, if there is any direct connection between these processes in respect to their light dependence. The results of Burström speak in favour of such a possibility, while the experiments by Tottingham et al. rather point in the opposite direction. In irradiation experiments with wheat plants these workers were able to show that by adding a small amount (4 per cent) of short-wave light from a carbon arc lamp with the largest increment of light intensity in the region between 3100 and 4000 Å, but a still quite substantial one between 4000 and 5000 Å, to the radiation from a tungsten incandescent lamp, nitrate assimilation (or rather absorption from the nutrient medium) could be raised by up to 68 per cent. No similar effect of short-wave light on photosynthesis is known.

Experiments were conducted at a restricted number of wave length regions from different parts of the visible spectrum. In addition experiments were done in white light from a incandescent lamp and in darkness. Usually every experiment was repeated six times.

From the beginning the intention was to perform all experiments at one single light intensity, viz. 22800 erg/cm². sec. As certain filters aged considerably and as the output of the mercury lamp, used for generating blue light, decreased continuously, the desired high value could not always be obtained. Later on this rather proved to be an advantage as it was possible at least for some wave lengths to get curves showing the dependence of assimilation on light intensity.

Plant material and cultivation

Wheat plants from a pure line, Svalöfs Progress, were generally used except for some homogeneity tests. The seeds which were treated with a sublimate-formaldehyde solution (0.1 per cent of each), were soaked in tap water for 24 hours at 25° C and then germinated on moist filter paper in large Petri dishes. After 48 hours in a dark-room at 25° C, as uniform plants as possible were selected and planted on small plexiglass disks, 17 plants on each. Four of these disks were put into a 1-liter glass beaker containing the following nutrient solution:

Table 1. Nitrate and chlorophyll content in different parts of wheat leaves during varied growing conditions. NO_3 =nitrate concentration in mmol/g fresh-weight. Chl.=chlorophyll concentration in mg/g fresh-weight. Segment 1=tip, segment 6=base. Length of segment 20 mm.

Segment	1		2		3		4		5		6	
Substance determined	NO_3	Chl	NO_3	Chl	NO_3	Chl	NO_3	Chl	NO_3	Chl	NO_3	Chl
Var. »Progress». 20° C Incandescent lamp	0.085	2.16	0.113	—	0.118	1.67	0.133	1.58	0.145	1.41	0.142	1.04
Var. »Eroica». 20° C ... Incandescent lamp	0.076	2.12	0.101	1.78	0.107	1.30	0.111	1.27	0.124	1.15	0.124	0.75
Var. »Eroica». 25° C ... Fluorescent lamp	0.029	2.76	0.056	2.65	0.078	2.28	0.082	2.02	0.090	1.73	0.102	1.28

KNO_3 1/500 mol, $\text{Ca}(\text{NO}_3)_2$ 1/250 mol, KH_2PO_4 3/1000 mol, Na_2HPO_4 1/1000 mol, MgSO_4 1/1000 mol, MnSO_4 1/50000 mol, Fe-citrate 1/50000 mol, ZnSO_4 1/200000 mol, H_3BO_3 1/200000 mol and $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$ 1/500000 mol. pH=about 6.3.

The beakers were placed in a photothermostat with 4 fluorescent lamps (Philips »Daylight», 100 W) and were allowed to remain there for 6 days at 25° C. Light was switched on between 5.00 a.m. and 9.00 p.m. The nutrient solution, which was changed after 4 days, was well aerated.

Homogeneity

An indispensable condition for the use of the experimental methods described below was the availability of an extremely homogenous plant material. Such a material at first proved to be rather difficult to obtain despite the fact, that all external factors, including air moisture, were held constant during cultivation. The reason was that the nitrate content in different parts of the wheat leaf shows a great variation. As appears from table 1 the nitrate concentration is lowest at the tip of the leaf and then rises continuously to the base, while the chlorophyll concentration behaves in the opposite way. It therefore proved necessary to use exactly the same part of the leaves. In all subsequent experiments a 55 mm long section was used, its cut ends being situated 18 resp. 73 mm from the tip. 14 sections were included in every experiment and table 2 shows that the degree of homogeneity in such a selected material was quite satisfactory.

Sugar content

To make sure that sufficient amounts of substrate for respiration were present in the leaves at the beginning of the experiments, reducing and not reducing sugars were determined in several cases. The analyses were performed according to the method of Philipsson (1943). Reducing sugar was expressed as mmol glucose/g freshweight, non-reducing sugar as mmol sucrose/g freshweight. Table 3 shows the results from 4 analyses.

Apparently the hexose concentration was very constant during the first three

Table 2. *Homogeneity test.* Nitrate content in samples of wheat leaves taken from the same culture. Values expressed as mmol NO₃/g fresh-weight.

Culture Sample	I	II	III
1	0.137	0.121	0.130
2	0.138	0.119	0.130
3	0.137	0.120	0.132

tests, while the concentration of sucrose varied considerably more. This tallies well with earlier results by Jones (1936) and Burström (1943) and obviously means that sucrose mainly serves as reserve material. As long as there are appreciable amounts of sucrose left, any shortage of respiration material is consequently not likely to occur, and not until sucrose is almost entirely consumed the immediate respiration substrate, i.g. the hexoses, begins to decrease. This is clearly shown by the last column of table 3, where the results of an analysis of plants, that have been held in darkness for 22 hours, are represented.

Arrangement of apparatus

Detached wheat leaves were placed in an assimilation chamber and irradiated with light of different wave lengths. Air with a known content of carbon dioxide was led through the chamber, after which the air was analysed for remaining carbon dioxide and the intensity of photosynthesis was calculated. The intensity of nitrate assimilation was obtained by determining the nitrate content in the leaves after finishing the experiment and comparing it with the content in a parallel sample, taken at the starting moment.

Two dark-rooms were available for the experiments. One of them was exclusively used for the light installations, including a heat absorption cuvette, while the other contained the rest of the apparatus. The light from the lamp in the former room could be sent into the latter through a hole in the wall between the two rooms. Appropriate light filters could be inserted in the light beam by means of a filter-holder just in front of the hole. The arrangement of the complete apparatus is schematically shown in figure 1.

The entire room to the left of the wall was temperature-regulated and held at a constant temperature of 24° C. Air with normal CO₂-content (c:a 0.04 vol%) was

Table 3. *Sugar content of wheat leaves.* Samples 1—3 are taken immediately before an irradiation experiment, sample 4 after 22 hours in darkness. The concentration is expressed as mmol/g fresh-weight.

Sample nr.....	1	2	3	4
Hexose	0.0115	0.0100	0.0104	0.0049
Sucrose	0.0098	0.0094	0.0028	0.0011
Totally	0.0213	0.0194	0.0132	0.0060

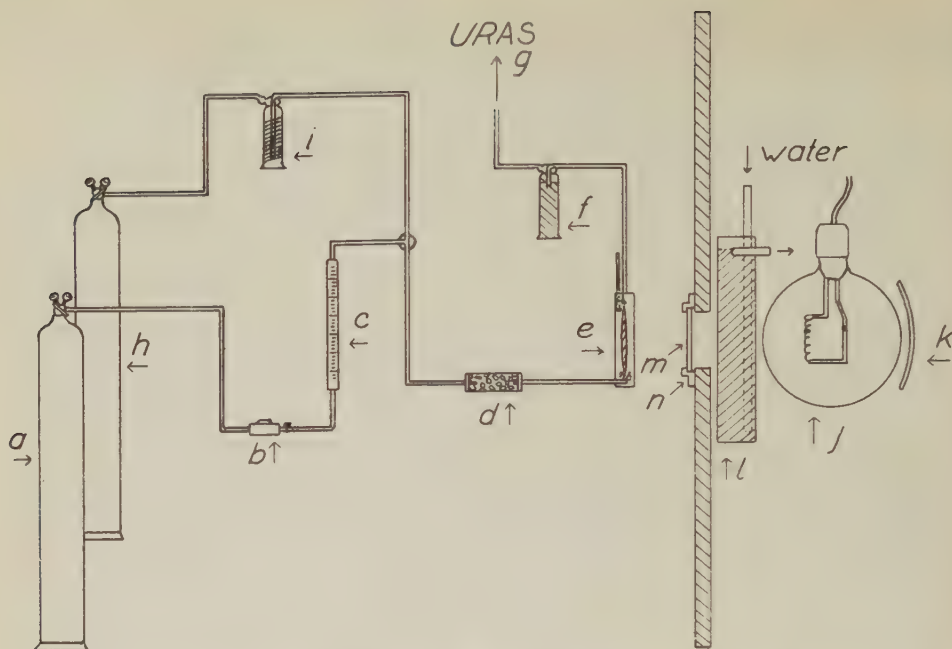


Figure 1. Arrangement of the experiments. Explanation is given in the text. The components are not drawn to scale.

kept in a steel cylinder (a) and from there led via a pressure regulator (AGA-TRAF-100) (b), a flowmeter (Rotameter type 100) (c) and a humidification device (glass tube filled with wetted earthenware pieces) (d) to the assimilation chamber (e). From the latter the air passed a drying apparatus (gas wash-bottle with conc. sulfuric acid) (f) and finally entered a carbon dioxide analyser (URAS from Badische Anilin und Sodafabrik) (g). For setting the zero, nitrogen gas from another steel cylinder (h) was used. The gas was led through a gas wash-bottle with alkali (i) into the assimilation chamber and from there to the analyser. The light-source (j) was either a tungsten incandescent lamp (Luma projector type B, all filaments in one plane, 3000 W) together with a concave mirror (k) or a water-cooled super high pressure mercury lamp (Philips SP 500 W, directed radiation). In order to eliminate the heat radiation from the light sources a 12 cm thick layer of running tap water (l) was inserted between these and the assimilation chamber. The remaining heat radiation was reflected by the colour filters or, if white light was used, by a Nife heat absorption filter and a Galflex heat reflexion filter. The colour filters (m), which were of the interference type, were mounted on a holder (n) in front of the hole in the wall. The holder, too, was cooled with running water.

Assimilation chamber

The construction of the chamber is illustrated by figure 2. It was made entirely by Perspex glass except for the removable front side, which consisted of 3 mm

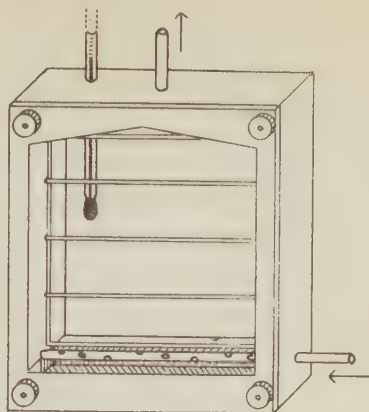


Figure 2. *The assimilation chamber. Scale 1:2.*

thick mirror glass. At the upper part of the chamber a thermometer was fixed in such a way that the bulb came just behind the assimilating leaves. Furthermore there were two hooks supporting a simple frame of music-wire. To this frame the leaves were attached with three thin rubber strings. The air was conducted into the chamber from below through a manifold with 10 holes and out of it through a single tube at its top. Smoke tests showed that the air stream became uniformly dispersed in this way. 14 leaves were chosen for every experiment and were attached to the frame side by side. The basal part of each leaf dipped into a few mm of water at the bottom of the chamber and therefore the area, exposed to the light, was a square with 50 mm long edges. Sample tests showed only neglectible diffusion of nitrate from the leaves to the small amount of water at the bottom of the chamber.

Colour filters

The choice of filters constituted a rather difficult problem, as on one side a highly monochromatic light was desired, but on the other side a sufficiently high intensity had to be obtained. Commercial interference filters, which are the best ones from the first point of view, usually have so narrow bands that the second condition in most cases cannot be fulfilled. Fortunately there was an opportunity to take over a number of interference filters with extra wide bands. These filters were manufactured by cand. mag. A. Hermansen at the Physical Laboratory, Royal Vet. and Agr. College, Copenhagen. For the experiments 5 filters were used with their optical centres at 4300, 4700, 5200, 6150 and 6550 Å respectively. Other characteristics of the filters may be evident from figure 3.

Measurement of light intensity

All intensities are expressed in absolute units, i.e. erg/cm². sec. The radiation was measured with a Kipp & Zonen vacuum thermocouple type D 1 together with a Multiflex mirror galvanometer MG 3. The instrument was calibrated for the different wave length regions by placing the thermocouple together with the corresponding

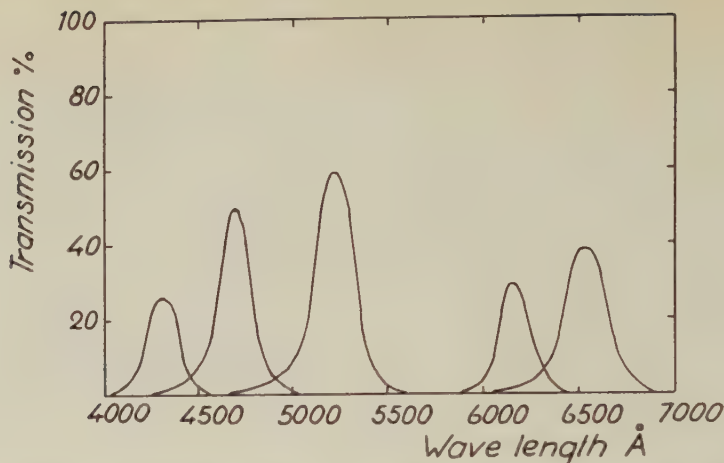


Figure 3. *Spectral transmission curves of the interference colour filters.* The data have been obtained by means of a Beckman spectrophotometer.

colour filter and a Calflex heat reflexion filter on a certain distance from a tungsten ribbon lamp with known emission in the different wave length regions (Hansson 1951), measuring the absorbed energy and comparing it with the emitted one, calculated from the data of the lamp. The thermocouple proved to be equally sensitive over the whole visible region.

The measurements in connection with the assimilation experiments were performed with the thermocouple mounted on the place of the assimilation chamber with the junction in the plane of the leaves and the front glass plate inserted in the light beam. The desired light intensity was achieved by moving the thermocouple to an appropriate distance from the lamp.

Determination of nitrate

The concentration of nitrate in the wheat leaves was determined by means of a procedure, consisting of a combination of the methods of Burström (1942) and Jones and Underdown (1953). The principle of the analysis is that the nitrate is extracted and adsorbed on an anion exchanger, after which it is eluted and the concentration determined colorimetrically according to the phenoldisulphonic acid method. In detail the analysis is performed as described below.

About 0.5 g fresh material is cut to small pieces, ground in a mortar and boiled slowly for 10 min. with 40 ml distilled water. After cooling, 0.5 ml 89 per cent H_3PO_4 is added and the volume made up to 50.00 ml with distilled water in a volumetric flask. The mixture is then filtered and 40.00 ml of the filtrate is allowed to flow through an anion exchanger (Amberlite IR-4B, 15×1 cm) at a rate of 1 drop in 2 sec. The ion exchanger must be in the hydroxy-form, which is generated by treating with 4 per cent NaOH. Organic substances and cations are washed out with 100 ml distilled water, whereupon the anions are eluted with 25 ml 4 per cent NaOH. The ion exchanger is thus automatically prepared for the next sample. It

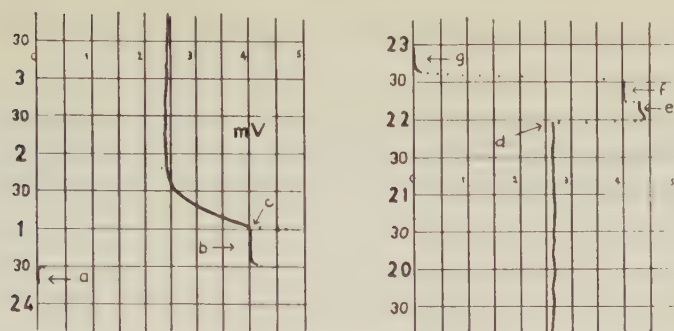


Figure 4. *Initial and final recordings of the URAS-carbon dioxide analyser during an experiment with white light. Explanation of the symbols is given in the text.*

is finally rinsed with 50 ml distilled water and the water is combined with the eluate. A small amount of activated charcoal (Supra-Norit or something alike) is added in order to absorb the yellow substances that are formed during the eluation and which have a disturbing effect on the following colorimetric determination. No adsorption of nitrate to the charcoal could be observed. After standing over night in a refrigerator the sample was filtered and distilled water added to 100.00 ml. 3.00—15.00 ml (depending on nitrate conc.) of the filtrate are evaporated in a small glass dish on a water bath. The evaporation does not require any supervision as neither sugars nor hydrogen peroxide are present. Larger amounts of chloride in the sample have to be removed in some way, i.g. by precipitating with silver sulphate. After evaporating, 2 ml of phenoldisulphonic acid are added and the mixture is allowed to stand for 10 min. It is then transferred to a 50 ml volumetric flask diluted with 25 ml distilled water added in small portions. Finally 15 ml 25 per cent NH_3 are added, the flask is cooled in cold water and filled up to the mark with distilled water. The intensity of the colour is determined with a suitable instrument, i.g. a Klett-Summerson photoelectric colorimeter. Accuracy about 1 per cent.

Determination of carbon dioxide

The carbon dioxide uptake of the wheat leaves was registered continuously during the whole experiment by means of an URAS-carbon dioxide analyser. A detailed description of the construction and use of this instrument can be found in the works of Egle and Ernst (1949), Huber (1950), Strugger and Baumeister (1951), Egle and Schenk (1951, 1952) and Baumeister (1952). Every 30 sec. a Hartmann & Braun automatic recorder registered the deviation of a galvanometer, coupled to the analyser. The paper in the recorder was graduated from 0—5 mV with 50 scale lines in all so that 1 mV approximately corresponded to 0.01 vol% CO_2 . It was possible to read $\frac{1}{4}$ scale line and consequently the reading accuracy was $\frac{1}{40}$ mV or about 0.00025 vol% CO_2 . As the air in the cylinder had a carbon dioxide content of 0.04 vol%, changes less than 1 per cent of the initial concentration could be detected. The analyser was calibrated by simultaneous determination of the carbon dioxide content in the air with a gravimetric method. A known volume of air was allowed

to stream through an absorption vessel, filled with Ascarite+Dehydrite (Mg-perchlorate).

In figure 4 a paper strip from the recorder is reproduced, showing the initial and final stages from an experiment with white light. Actually the strip was much longer, but the central part has been excluded for practical reasons. The apparatus was set to zero by conducting carbon dioxide-free nitrogen gas through it (a). Thereupon air from the cylinder was allowed to flow through the instrument for half an hour in order to adjust it for a reading, corresponding to the carbon dioxide content of the air (b). The experiment then was started by connecting the assimilation chamber with the air conduct from the cylinder (c). After 22 hours the experiment was finished by switching off the lamp (d), whereupon the leaves had to remain in darkness for 15 min., during which time the dark-respiration was determined (e). The leaves were then removed and the instrument again checked with air from the cylinder (f) and with nitrogen (g).

The carbon dioxide assimilation was calculated in the following way. From the curve the decrease of carbon dioxide concentration was obtained. As this value corresponds to the apparent assimilation, it was necessary to add the value of respiration in order to get the real assimilation. The question, if respiration in light is of the same magnitude as in darkness, has for long time been an unanswered one, but recent experiments on *Chlorella* by Brown (1953) with isotopically enriched oxygen, strongly indicate that at least in this material there is no difference. It has been thought reasonably fair to assume that the same conditions prevail also for wheat leaves.

Respiration in darkness has to be determined before and after the experiment. The numerical value of respiration before the experiment proved, however, to be so low, that no significant differences could be obtained between them. A standard value therefore could be used all the time. Respiration after the experiment on the contrary varied considerably, probably depending on more or less strong formation of assimilates. It is also evident from the figure that immediately after switching off the lamp there was a strong extra production of carbon dioxide, which only lasted for a few minutes. The carbon dioxide concentration then again fell slightly and stabilized at a constant level, yet somewhat higher than at the beginning of the experiment. This extra- CO_2 production has been observed by earlier workers, i.g. van der Veen (1949). The cause is not known with certainty, anyhow, there is no reason to suspect any after-effect of a presumptive light-respiration. The values for respiration before and after the experiment were connected with a straight line and from this line as a basis the changes in carbon dioxide content, i.e. the real assimilation, were computed for every 30 minutes. Finally also the mean value for the assimilation during the whole experiment was determined.

Chlorophyll determination

The chlorophyll was extracted with boiling ethyl alcohol. The solution then was made up to a known value by the same solvent and the transmission determined with a Beckman spectrophotometer at 6550 Å. For a rough estimation of the chlorophyll-a the extinction coefficient was set to 6.6×10^4 . The quotient chlorophyll-a/chlorophyll-b was assumed to be 2.5.

Results and Discussion

Carbon dioxide assimilation

The influence of wave length on the light reactions of photosynthesis has been investigated many times, but not until the last decennia reliable, quantitative results have been presented. Reviews of more important works on this subject have been written by i.a. Gabrielsen (1940) and Rabinowitch (1951).

According to Einsteins law of photochemical equivalency a given number of light quanta, absorbed by a certain pigment, should always produce the same photochemical effect, irrespective of the wave length of the light. In the case of photosynthesis one would therefore expect to get equal assimilation in the different wave length regions, provided only that the light intensities were measured in absolute units and adjusted to the same number of quanta. This, however, proved not to be true. There are several reasons for this phenomenon. Thus the degree of absorption varies with the wave length of light, and besides, it is very difficult to determine the real absorption in the pigments accurately, owing to useless absorption in cell-walls, different degree of reflexion etc. Furthermore there often are several pigments in the photosynthesizing organ, which may compete or act as optical screens and finally the quantum requirement may not be equal for all wave lengths. If the photosynthetic yields for the same number of quanta are represented as a function of wave length, one thus obtains a curve, the so called action spectrum, and not a straight line. This curve may, depending on the experimental arrangement, more or less parallel the corresponding absorption curve of the photosynthesizing organ. The parallelism may be very close for thin organs and with low light intensities, but will be leveled out to a considerable extent if the organ is thick or the intensity high. (For further discussions on this subject see Rabinowitch 1951).

The paper of Emerson and Lewis (1943) affords a good illustration on this point. By irradiating a thin *Chlorella* suspension with light of different wave lengths, they got an action spectrum that was very similar to the absorption spectrum except in the region between 4000 and 5000 Å, where the efficiency was somewhat too low. The curve had two peaks and the minimum efficiency was obtained at 5400 Å. If they used a thick suspension, the absorption was total for all wave lengths, but the quantum yield nevertheless had a very definite minimum at 4900 Å. Emerson and Lewis explained this decrease in the blue region by assuming that carotinoids had absorbed a certain proportion of the incident light. Calculations, based on the absorption spectrum of the carotinoids showed, however, that this proportion was not entirely inactive in photosynthesis, but its efficiency was reduced to about 50 per cent. The study of the course of the action curve can thus give valuable information on the occurrence, and sometimes of the quantitative aspects, of different pigments in the investigated system.

The results from the present investigation can be represented in two ways. Either the mean values of the carbon dioxide assimilation, as calculated every 30 min., are stated as in figure 5, or the maximal values (or rather the means of the 5 highest values) are chosen and curves as in figure 6 are

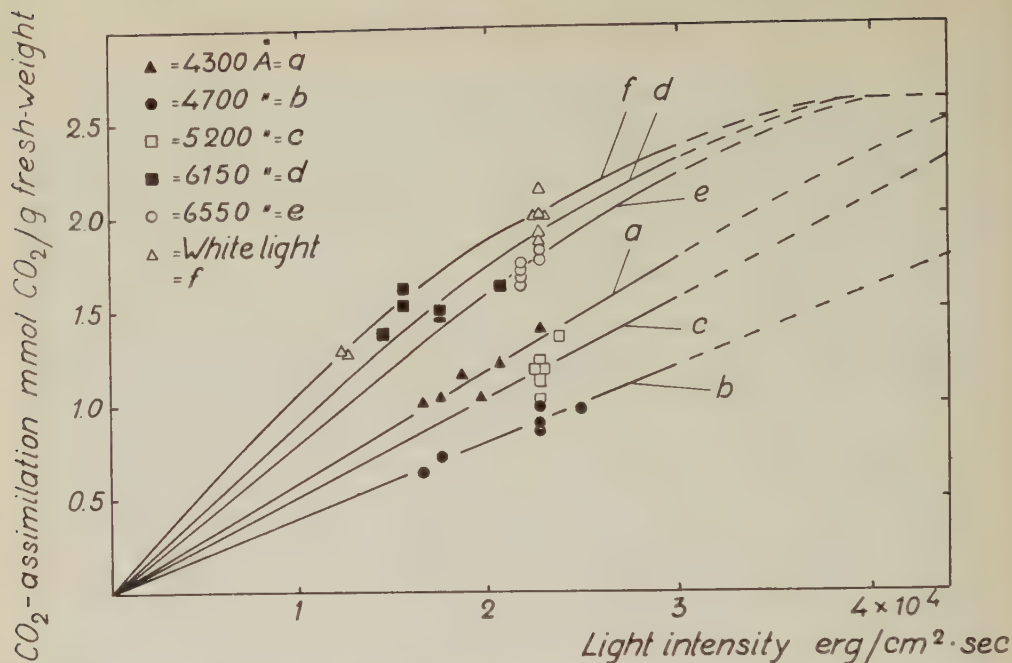


Figure 5. Mean values of carbon dioxide assimilation at different wave lengths and light intensities.

obtained. The corresponding curves seem very alike, yet they are not quite identical as the assimilation rate did not remain constant in all wave length regions. The curves in figure 5 are to be used in comparisons with nitrate assimilation, the ones in figure 6 in an analysis of the photosynthetic process. As already mentioned before, these curves may only be regarded as approximative ones, and this is particularly the case with those for 6150 and 6550 Å. The shape of the curves seems to be in very good agreement with the results of Gabrielsen (1940) with *Sinapis*, according to which the maximal assimilation for all wave lengths (including white light) asymptotically approaches one and the same value.

As shown in table 4, the assimilation, especially in the red region, did not remain constant but decreased gradually during the course of an experiment, while no such observation could be made in blue and in white light. The reason for this decrease is not known with certainty. A stomatal effect is possible, but not very probable, owing to the regular course of the continuously registered alteration. Another possibility is that the presence of short-wave light is necessary for the formation of some essential intermediate product in photosynthesis and that monochromatic red light therefore in

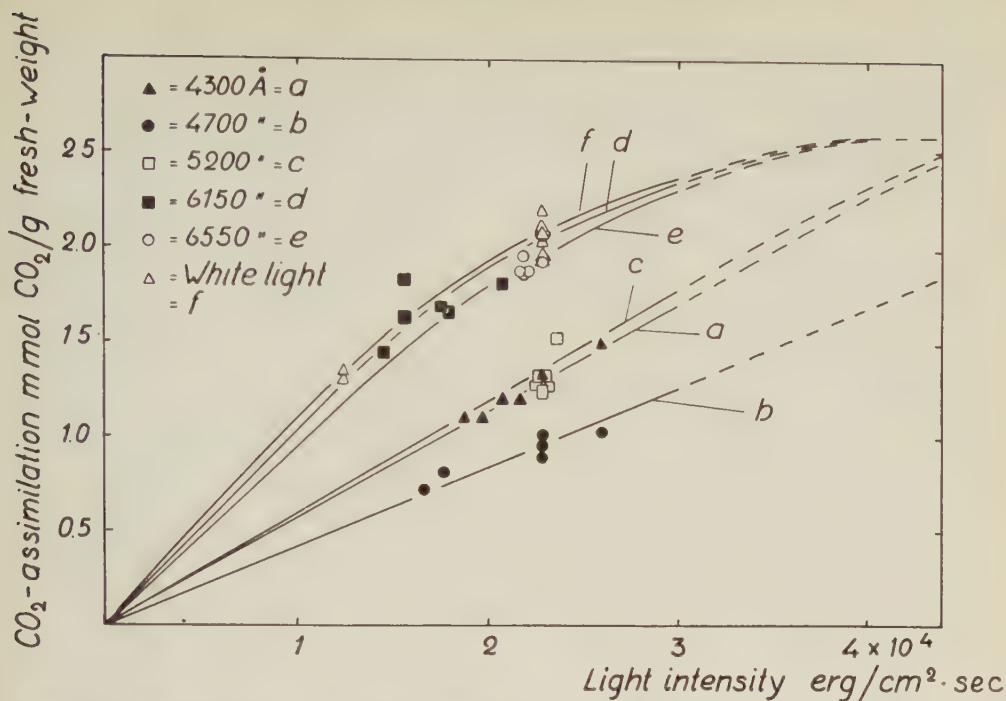


Figure 6. Maximum values of carbon dioxide assimilation at different wave lengths and light intensities.

spite of its high momentary effect on photosynthesis in the course of time cannot maintain a high rate of assimilation because this intermediate product is relatively soon exhausted. A remarkable fact is that the intensity of assimilation in white light from the incandescent lamp is only slightly altered during 22 hours though the blue proportion in this light must be very small.

In this connection a paper of Warburg, Krippahl and Schröder (1954) deserves to be mentioned. These workers claim to have shown a catalytical effect of blue-green light on the photosynthesis of *Chlorella*. During their experiments in monochromatic red light ($\lambda=6440$ Å) the assimilation intensity kept constant only for a few hours after which time it decreased slowly, but steadily. Addition of a small amount of white or blue-green light ($\lambda=4680-5090$ Å) was enough to ensure a constant assimilation rate. Warburg et al. interpreted this effect by assuming the activation of an enzyme by a carotinoid, inasmuch as these pigments have their maximal absorption just in the blue-green region. Attempts by others, e.g. Bassham, Shibata and Calvin (1955) to confirm these findings have failed, however.

It would be tempting to apply the same explanation to the present results with wheat, but there are certain difficulties. The wave length region about 4300 Å seems to be as effective as that about 4700 Å in maintaining constant

Table 4. *Decrease in carbon dioxide assimilation intensity during 22 hours' irradiation with different light qualities. The decrease is expressed in per cent of the initial intensity.*

Wave length (Å)	4300	4700	5200	6150	6550	White light
Experiment 1	0.0	0.0	11.9	15.6	20.3	4.3
» 2	0.0	0.0	7.0	22.6	12.3	4.9
» 3	0.0	0.0	7.1	10.7	6.3	5.9
» 4	0.0	0.0	18.0	19.0	7.5	8.3
» 5	0.0	14.3	14.0	9.3	14.3	7.5
» 6	6.5	4.3	22.0	5.7	12.7	4.5
Mean value	1.1	3.1	13.3	13.8	12.2	5.9

assimilation, and in the former region the carotinoids show a rather weak absorption. Measurements of the absorption by the yellow, ether-soluble pigments from the wheat leaves gave three maxima at 4250, 4500 and 4700 Å respectively. In the living cell these maxima are shifted to the red by some 150 Å, (owing to linkage to a protein), if the pigment is a carotinoid (Rabinowitch 1951). The values in such a case will become 4400, 4650 and 4850 Å instead. Indubitably there will be a considerable absorption also at 4300 Å and it is possible, that this absorption will be sufficient to induce the catalytical effect. Another explanation, already discussed by Warburg et al. is, however, that other pigments than carotinoids are involved. The flavines for instance have an absorption spectrum which in the visible region is very similar to that of the carotinoids. Warburg et al. abandoned this alternative on the plea that no light-activation of a flavin enzyme ever had been shown. This statement of course does not exclude the possibility that there nevertheless exists such an activation. As this problem will be treated further in connection with the light effects on nitrate reduction it is referred to this discussion.

As already mentioned the assimilation values ought to be compared for equal numbers of incident quanta. This can, however, only be done as long as the points are on the linear, ascending part of the light intensity curve. For that reason the value 8.05×10^{-9} einstein/cm². sec has been chosen as a basis, which corresponds to 2.25×10^4 erg/cm². sec at 4300 Å and to 1.46×10^4 erg/cm². sec at 6550 Å. The assimilation values thus obtained from the intensity curves are represented graphically in figure 7 together with the spectral curves of the quantum yield of a dense and the active absorption of a thin *Chlorella* suspension. The result is rather remarkable. The action spectrum of photosynthesis does not appear to follow the absorption spectrum of the living leaf (figure 8) or the thin *Chlorella* suspension, but resembles much more the quantum yield curve of Emerson and Lewis for a dense algal suspension. This is probably connected with the partial absorption of the radiation by pigments other than chlorophyll (and with lower photosynthetic

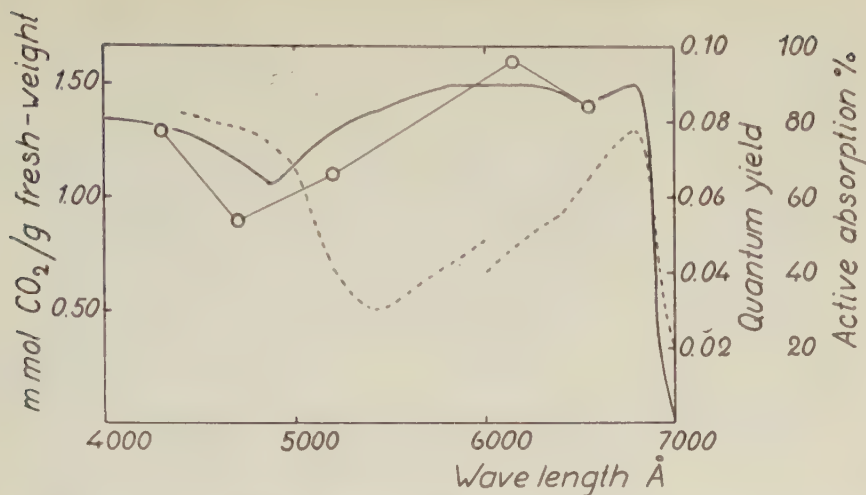


Figure 7. Comparison between the action spectra of photosynthesis in wheat leaves and in different *Chlorella* suspensions.

○—○ wheat leaves
 — dense *Chlorella* suspension (From Emerson & Lewis 1943)
 - - - - thin " " " " " " " "

efficiency), especially between 4500 and 5500 Å. Most striking is perhaps the observation that the minimum of photosynthesis lies at 4700 Å, in good agreement with the data of Emerson and Lewis, and not at 5200 Å, where it would have been reasonable to expect it with guidance of the absorption spectrum of the leaf. Probably the true assimilation minimum, dependent only on the unequal absorption in the chlorophyll, is masked by strong absorption in yellow pigments. As there is an ample occurrence of carotinoids in the leaves, and as the minimum of assimilation coincides with their absorption maxima, there is good reason to suspect that absorption in these pigments is responsible for the low assimilation intensity in the blue-green region. The action curve of photosynthesis in wheat also shows a slight dip at 6550 Å, comparable with a corresponding depression in the curve of Emerson and Lewis, but the significance of this dip is questionable.

The results with wheat, quoted above, give a quite different picture of the action spectrum of photosynthesis than an earlier investigation with the same material by Hoover (1937). He compared the assimilation at equal absolute intensities, but his results have been quantized by Burns (1938). They are reproduced in figure 8, together with the absorption curve of a living leaf. Apparently this action spectrum does not agree very well with the absorption spectrum of the living leaf and still less with the values from the present investigation. Instead there is good agreement with the absorption and assimilation of a thin *Chlorella* suspension. The question then rises, if the differences between Hoover's results and the present ones can be

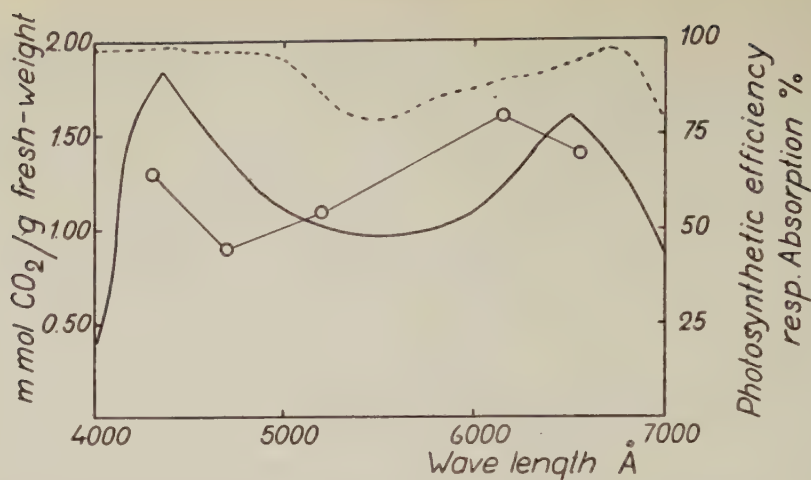


Figure 8. Two action spectra of photosynthesis in wheat leaves, determined according to different methods, as compared with the absorption spectrum of a living leaf.

— Action spectrum from Hoover 1937, quantized by Burns 1938.

○—○ Action spectrum from the present investigation.

----- Absorption spectrum of the living leaf. (Determined according to Shibata, Benson & Calvin 1954).

explained by differences in the chlorophyll content in the experimental material. Already Gabrielsen (1940) pointed out that assimilation curves with two peaks are obtained when using plants with low chlorophyll content, e.g. most green algae, but that materials with a high concentration of chlorophyll, as nearly all higher plants, give curves with a single peak in the red. The only exception to this rule are the results with wheat. It then appears quite reasonable to guess that the chlorophyll content in these experiments has been particularly low. This opinion is supported by the fact that the wheat plants in both cases had been reared in artificial light, which is known to cause poor chlorophyll formation. Rough estimations of the chlorophyll content in my own material also gave considerably lower values than reported from other angiosperms, as shown in table 5. As Hoover worked with incandescent lamps instead of day-light fluorescent lamps, his material should have suffered of a still lower chlorophyll content (compare table 1). This might be the main reason for the differences between our results.

In studying the curves in figure 6 another peculiar phenomenon is observed, namely that the assimilation in white light is as strong as that in red light.

Table 5. Determination of chlorophyll (*a* + *b*) in some different plants.

Ulva (Seybold & Egle 1937 & 1938)	0.49	mg/50	cm ²
Triticum (Stoy 1955)	2.0	»	»
Fraxinus, shade leaves (Gabrielsen 1940)	2.4	»	»
» , sun leaves	3.3	»	»

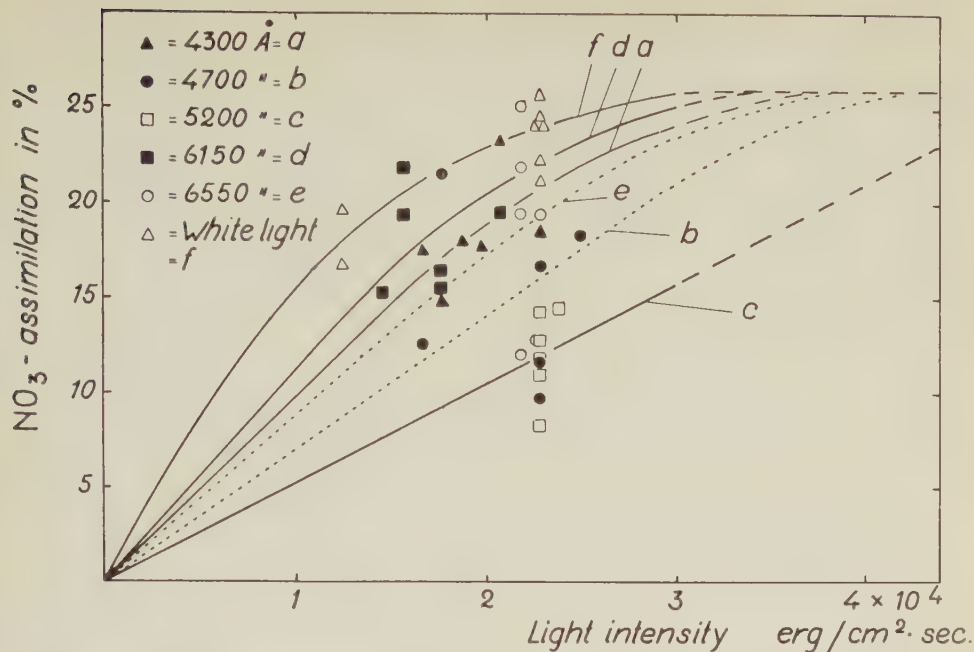


Figure 9. Nitrate assimilation at different wave lengths and light intensities.

One would expect the former to have a lower value, as there must be fewer quanta compared with the latter. From experiments with *Sinapis* such results also are recorded (Gabrielsen 1940). However the optical density and the more or less complicated anatomical structure of the material exerts a great influence on the optical properties of the light-absorbing system, and therefore the results must not necessarily be identical in all cases.

Nitrate assimilation

The results are represented graphically in figure 9. It is obvious at a first glance that there is a considerably greater scattering of the values than was the case with photosynthesis. This is especially evident at 4700 and 6550 Å. These curves therefore are dotted in order to stress this uncertainty and it should be kept in mind that the following comparison between the results on nitrate assimilation and photosynthesis is only preliminary in nature. The reasons for the greater variation, compared with photosynthesis, may be of several kinds. Firstly the absolute values, expressed in mmol/g fresh-weight, are almost 100 times lower for nitrate assimilation than for photosynthesis and consequently the errors of analysis will play a relatively greater

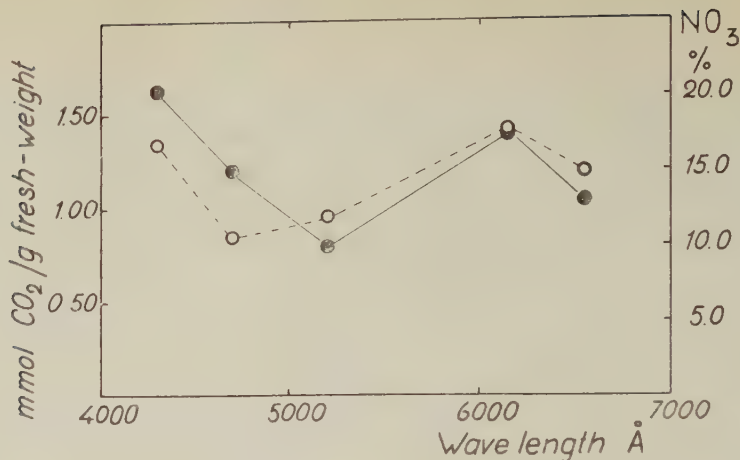


Figure 10. Comparison between the action spectra of nitrate- and carbon dioxide assimilation.

- Nitrate assimilation.
 ○—○ Carbon dioxide assimilation.

role in the former case. Secondly the action curve may have a steeply ascending or descending part, with a greater scattering of the assimilation values as a consequence.

Also for the nitrate assimilation the comparison between the different wave lengths has been done at the same number of incident light quanta, i.e. 8.05×10^{-9} einstein/cm². sec. The values for nitrate and (mean) carbon dioxide assimilation are both represented in figure 10. The curve for nitrate assimilation has two peaks too, but the highest one is situated in the short wave region, while the minimum lies at 5200 Å and not at 4700 Å as was the case with photosynthesis. These interesting findings will be further discussed in the next section.

Nitrate reduction in white light is at least as strong as in anyone of the monochromatic regions, furthermore the values appear to be less scattered than in these. The course of the intensity curves seems to be approximately the same as for photosynthesis.

The question if nitrates can be formed in darkness in detached leaves, has been disputed for a long time and still no conclusive arguments have been presented for or against. The literature on this subject has recently been reviewed in a paper of Munsche (1955). Many of the older works suffer under serious defects, as inhomogeneity of the material and insufficient precision in the methods of analysis. In the present investigation, 5 experiments were made with plants held in darkness for 22 hours. In 4 cases the

Table 6. *Changes of nitrate content in wheat leaves after 22 hours in darkness. The changes are expressed as per cent of the initial nitrate concentration.*

Experiment	Change (%)
1	+ 1.7
2	+ 3.1
3	+ 2.9
4	+ 2.2
5	— 1.4
Mean values	+ 1.7

nitrate content increased, while it decreased in the 5:th one. The average increase for all 5 experiments was 1.7 per cent (table 6).

Comparison between carbon dioxide and nitrate assimilation

The main result of the investigation thus is that it has been possible to show that the light intensity curves of the carbon dioxide- and the nitrate assimilation are almost identical in white and red light (possibly white light gives a somewhat stronger nitrate reduction), but that in light of shorter wave lengths nitrate assimilation is obviously promoted. This indubitably points towards the existence of a yellow pigment which is capable of transforming part of the absorbed energy into chemical one, especially active in nitrate reduction. At the moment it is not possible to determine the identity of this pigment. Its absorption in the visible part of the spectrum must lie between 4000 and 5000 Å and of known pigments carotinoids and flavines appear the most plausible ones.

Relatively little is known about the role of the carotinoids in the redox-processes of the cell, but as regards photosynthesis the general opinion seems to be that the carotinoids are able to transfer a greater or lesser part of the energy absorbed by them to chloróphyll-a, but that they themselves do not possess any capability to act as direct photocatalysts. The question then arises, if they have this ability in the case of nitrate reduction, but the results of the investigation do not speak in favour of this possibility. In comparing the curves for carbon dioxide and nitrate assimilation in figure 10 one gets the impression that a depression of the nitrate assimilation exists in the blue-green light in the same manner as for the carbon dioxide assimilation (see p. 977) but that in addition a stimulating influence is exerted by the light at 4300 and 4700 Å. There are at least four reasons that support the opinion that the activated pigment is of the flavine type and not a carotinoid:

1) The effect of light seems to be as strong at 4300 as at 4700 Å. The

absorption of the carotinoids (especially in the bound form) is however much stronger at the latter wave length.

2) Through the papers of Nason and Evans (1953) and Evans and Nason (1953), which described experiments with both lower and higher plants, it is established that at least the enzyme, that reduces nitrate to nitrite, is a flavoprotein with flavinadenin dinucleotide (FAD) as the prosthetic group. An activation of this enzyme through light is no doubt a conceivable possibility, but no such process has yet been proven.

3) In the phototropic reactions in higher plants the perception of the light impulse is brought about by means of a yellow pigment. This pigment is now with certainty shown to be riboflavin (Galston 1949, Galston and Baker 1949, Reinert 1952, Brauner 1953). The light action involves a photochemical oxidation of indolacetic acid (IAA). According to Brauner and Brauner (1954) this photooxidation has the character of an oxidative decarboxylation, a process in which the role of oxygen seems to be to reoxidise the reduced riboflavin. It is tempting to assume that the oxygen from the air can be substituted by oxygen from the nitrate. At all events the reduced riboflavin is not restricted to free oxygen as the only possible hydrogen acceptor as it also can reduce oxidised ascorbic acid (Brauner and Brauner 1954). In this connection it may be mentioned that ascorbic acid frequently has been assumed to participate in nitrate assimilation (Virtanen 1950).

4) In the already reviewed experiments of Tottingham et al., the added carbon arc light exerted its greatest effect in the region between 3100 and 4000 Å, while a less pronounced, but quite evident one was produced by the wave lengths up to 5000 Å. Riboflavin shows strong absorption in the long-wave ultraviolet and a somewhat lesser one between 4000 and 5000 Å, whereas the carotinoids only absorb in the latter region. These findings seems to provide evidence for the opinion that the yellow pigment is a flavin.

The possibilities discussed under point 2) and 3) differ from each other in one important respect. In the former case the pigment exists as an enzyme, i.e. bound to a protein, while in the latter one the flavin found in free form. This is of importance for their absorption- and action spectra, because the linkage to a protein is accompanied by a shift in the spectrum to somewhat longer wave lengths. The strong action of light at 4300 Å therefore speaks in favour of a reaction of the latter type, the data that are available are however too meager to allow definite conclusions. In this connection the attention should be drawn to the effect of short-wave light on the constant course of photosynthesis (see p. 974), there seems to be a striking resemblance between the light effects on this process and on nitrate assimilation.

As is evident from the experiments described above no reduction of nitrate takes place in darkness. This is however no general phenomenon, both chlorophyll-free organs like most roots and organs containing chlorophyll as the leaves of for instance tomato are able to reduce nitrate also in the absence of light. In these cases the necessary energy is received from respiration, probably with co-operation of ATP. As the light- and dark reactions seem to be distributed rather by chance within the plant (sometimes they even occur side by side), one gets the impression that these processes are identical in essential parts and that the only difference lies in the various ways in which the hydrogen donors, necessary for the nitrate reduction, can be generated.

Another unsolved problem is left, however, i.e. the role of carbon dioxide in nitrate assimilation. The chemical reactions discussed above ought to be possible even in the complete absence of this compound, but as was already mentioned in the introduction, Burströms experiments showed that even at a reduced carbon dioxide content the nitrate assimilation stops almost completely. To explain this result we must remember that assimilation not only means the reduction of the nitrate to the NH_2 -level, but also a fixation of the amino-radical (or a precursor to this) to a suitable carbon skeleton, i.g. an activated keto acid. The formation of such an acid is usually thought to be connected with respiration, but it seems very possible that these acids can be formed as intermediate product in photosynthesis as well. As respiration is not a uniform process in all plants or parts of plants but probably can proceed along several different lines, it seem reasonable to assume that not all of these lines can bring about the necessary combination of hydrogen donors and suitable carbon chains. In such a case this combination is not attained until photosynthesis is co-operating.

The discussions on the preceding pages could be concluded by the following working hypothesis: Nitrate is reduced both in light and in darkness by means of the same fundamental chain of reactions, the very difference lying in the mode of supplying energy and hydrogen donors for the reduction of the nitrate and carbon skeletons for the fixation of the amino radical (or a precursor to this). In *light* the reduction occurs photochemically and in addition to chlorophyll a yellow pigment seems to be specifically active in transferring the light energy. Some reasons speak in favour of this pigment being of the flavin type and not a carotinoid. Probably the necessary carbon compound is generated as an intermediate product in photosynthesis. In *darkness* on the other hand respiration energy is the driving force. In this case respiration also provides the carbon skeleton, presumably because this process in the dark-reducing organs proceeds along other lines.

Summary

The simultaneous reduction of carbon dioxide and nitrate in detached wheat leaves has been determined in five wave length regions from different parts of the visible spectrum, in white light and in darkness. The intensity of the CO_2 -assimilation was constant for 22 hours in white and short-wave light, but decreased with time in the long-wave region. Approximate light intensity curves were drawn and assimilation values, corresponding to equal numbers of incident quanta, were taken from the linear, ascending part of these curves. The action spectrum for CO_2 -assimilation showed two peaks at about 4300 and 6150 Å respectively and a minimum at 4700 Å. For NO_3 -assimilation there were also two peaks at the same wave lengths as for photosynthesis, while the minimum lay at 5200 Å instead. A comparison between the two processes showed, that white and long-wave light is as effective in NO_3 -assimilation as in photosynthesis, while short-wave light gave a higher yield for the former process. This is thought to be due to absorption in a yellow pigment, specifically effective in the reduction of NO_3 . In darkness a small increase in NO_3 has been observed. A working hypothesis regarding the differences between the assimilation of NO_3 in light and in darkness has been advanced.

The author wishes to thank Prof. H. Burström for suggesting the problem and for his never failing interest during the course of the investigation, Prof. H. Lundegårdh for giving me the opportunity to fulfill the experiments at Lund, Cand. mag. A. Hermansen for his kindness of letting me take over a number of interference filters, Fil. lic. N. Hansson for great help in calibrating the thermocouple and Civ.ing. V. Tullin for stimulating criticism and practical advices.

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References

- Arnon, D. J., Allen, M. B. & Whatley, F. R.: Photosynthesis by isolated chloroplasts. — *Nature* 174: 394. 1954.
- Bassham, J. A., Shibata, K. & Calvin, M.: Quantum requirement in photosynthesis related to respiration. — *Biochim. Biophys. Acta* 17: 332. 1955.
- Baumeister, W.: Zur Anwendung des Ultrarotabsorptionsschreibers für CO_2 -Assimilationsmessungen an abgeschnittenen Blättern im Laboratorium. — *Ber. Dtsch. Bot. Ges.* 65: 361. 1952.
- Brauner, L.: Untersuchungen über die Photolyse des Heteroauxins I. — *Zeitschr. f. Bot.* 41: 291. 1953.
- & Brauner, M.: Untersuchungen über die Photolyse des Heteroauxins. II. — *Zeitschr. f. Bot.* 42: 83. 1954.
- Brown, A. H.: The effects of light on respiration using isotopically enriched oxygen. — *Amer. J. Bot.* 40: 719. 1953.

- Burns, R.: Photosynthesis and the absorption spectra of plant pigments II. — Amer. J. Bot. 25: 166. 1938.
- Burström, H.: The colorimetric determination of nitrate in plant materials. — Sv. Kem. Tidskr. 34: 139. 1942.
- Photosynthesis and assimilation of nitrate by wheat leaves. — Ann. Agr. Coll. Sweden 11: 1. 1943.
- Dittrich, W.: Zur Physiologie des Nitratsumsatzes in höheren Pflanzen (unter besonderer Berücksichtigung der Nitratspeicherung). — Planta 12: 69. 1930.
- Egle, K. & Ernst, A.: Die Verwendung des Ultrarotabsorptionsschreibers für die vollautomatische und fortlaufende CO₂-Analyse bei Assimilations- und Atmungsmessungen an Pflanzen. — Zeitschr. f. Naturforschg. 4 b: 351. 1949.
- & Schenk, W.: Die Anwendung des Ultrarotabsorptionsschreibers in der Photosyntheseforschung. — Ber. Dtsch. Bot. Ges. 64: 180. 1951.
- Untersuchungen über die Reassimilation der Atmungskohlensäure bei der Photosynthese der Pflanzen. — Beiträge Biol. d. Pfl. 29: 75. 1952.
- Emerson, R. & Lewis, C.: The dependence of the quantum yield of *Chlorella* photosynthesis on wave length of light. — Amer. J. Bot. 30: 165. 1943.
- Evans, H. J. & Nason, A.: Pyridin nucleotide-nitrate reductase from extracts of higher plants. — Plant Physiol. 28: 233. 1953.
- Gabrielsen, E. K.: Einfluss der Lichtfaktoren auf die Kohlensäureassimilation der Laubblätter. — Kopenhagen (Einar Munksgaard). 1940.
- Galston, A. W.: Riboflavin-sensitized photooxidation of indoleacetic acid and related compounds. — Proc. Nat. Acad. Sci. 35: 10. 1949.
- & Baker, R.: Studies on the physiology of light action II. The photodynamic action of riboflavin. — Amer. J. 36: 773. 1949.
- Hansson, N.: An attempt to a photographic calibration of a tungsten ribbon lamp. — Ark. f. Astron. 1 (21): 223. 1951.
- Hoover, W. H.: The dependence of carbon dioxide assimilation in a higher plant on wave length of radiation. — Smithsonian. Misc. Coll. 95: No 21. 1937.
- Huber, B.: Registrierung des CO₂-Gefälles und Berechnung des CO₂-Stromes über Pflanzengesellschaften mittels Ultrarot-Absorptionsschreiber. — Ber. Dtsch. Bot. Ges. 63: 52. 1950.
- Jones, S. B. & Underdown, R. E.: Determination of nitrate in plant materials. — Analyt. Chem. 25: 806. 1953.
- Jones, W. W.: Respiration and metabolism in etiolated wheat seedlings as influenced by phosphorus nutrition. — Plant Physiol. 11: 565. 1936.
- Kessler, E.: Ueber die Wirkung von 2,4-dinitrophenol auf Nitratreduktion und Atmung von Grünalgen. — Planta 45: 94. 1955.
- Lease, E. J. & Tottingham, W. E.: Photochemical responses of the wheat plant to spectral regions. — J. Am. Chem. Soc. 57: 2613. 1935.
- Munsche, D.: Gibt es eine Nitrifikation in höheren Pflanzen? — Zeitschr. Pflanzenernährg. Düng. u. Bodenkunde 68: 1. 1955.
- Nason, A. & Evans, H. J.: Triphosphopyridine nucleotide-nitrate reductase in *Neurospora*. — J. Biol. Chem. 202: 655. 1953.
- van Niel, C. B., Allen, M. B. & Wright, B. E.: On the photochemical reduction of nitrate by algae. — Biochim. Biophys. Acta 12: 67. 1953.
- Philipsson, T.: Ueber die quantitative Bestimmung reduzierender Zucker. — Ark. f. Kemi 16 A 22. 1943.

- Rabinowitch, E. J.: Photosynthesis I. — New York (Interscience Publishers). 1945.
— Photosynthesis II: 1. — New York (Interscience Publishers). 1951.
- Reinert, J.: Ueber die Bedeutung von Carotin und Riboflavin für die Lichtreizaufnahme bei Pflanzen. — *Naturwiss.* 39 (2): 47. 1952.
- Seybold, A. & Egle, K.: Lichtfeld und Blattfarbstoffe I. — *Planta* 26: 491. 1937.
— — Quantitative Untersuchungen über Chlorophyll und Carotinoide der Meeresalgen. — *Jahrb. wiss. Bot.* 86: 50. 1938.
- Shibata, K., Benson, A. A. & Calvin, M.: The absorption spectra of suspensions of living micro-organisms. — *Biochim. Biophys. Acta* 15: 461. 1954.
- Strugger, S. & Baumeister, W.: Zur Anwendung des Ultrarotabsorptionsschreibers für CO₂-Assimilationsmessungen im Laboratorium. — *Ber. Dtsch. Bot. Ges.* 64: 5. 1951.
- Tottingham, W. E. & Lowsma, H.: Effects of light upon nitrate assimilation in wheat. — *J. Am. Chem. Soc.* 50: 2436. 1928.
— Stephens, H. L. & Lease, E. J.: Influence of shorter light rays upon absorption of nitrate by the young wheat plant. — *Plant Physiol.* 9: 127. 1934.
- Warburg, O., Krippahl, G. & Schröder, W.: Katalytische Wirkung des blaugrünen Lichts auf den Energieumsatz bei der Photosynthese. — *Zeitschr. Naturforschg.* 9b: 667. 1954.
— & Negelein, E.: Ueber die Reduktion der Salpetersäure in grünen Zellen. — *Biochem. Zeitschr.* 110: 66. 1920.
- van der Veen, R.: Induction phenomena in photosynthesis. I. — *Physiol. Plant.* 2: 217. 1949.
- Vishniac, W. & Ochoa, S.: Fixation of carbon dioxide coupled to photochemical reduction of pyridine nucleotides by chloroplast preparations. — *Journ. Biol. Chem.* 195: 75. 1952.
- Virtanen, A. I.: Utilization of the nitrate ion by plants and its relation to the assimilation of the ammonium ion and molecular nitrogen. — *Acta Agr. Scand.* 1: 1. 1950.

Root and Shoot Elongation Activity of Some Naphthalene Compounds

By

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It is almost unanimously assumed in the current discussion of the relation between structure and auxin activity that 1-naphthyl-acetic acid behaves as a genuine auxin (cf. however Street 1955). A comparison between the four 1- and 2-naphthyl-acetic and oxyacetic acids (Burström 1955 a) has shown that 1-naphthyl and 2-naphthoxy-acetic acids are auxins, and 1-naphthoxy and 2-naphthyl-acetic acids are antiauxins. This behaviour has been explained by a principle formulated by Jönsson (1955). It implies that a substance of the general structure of auxins has auxin activity if (1) the side chain can form a pseudo-ring in the plane of the ring system, (2) the carboxyl group of the pseudo-ring is located near the centre of the whole system, (3) at least one side of this complex is flat, devoid of protruding groups others than oxygen or hydroxyl, and (4) oxygen and hydroxyl of the carboxyl are perpendicular to the ring. These requirements are fulfilled by the two mentioned auxins, but requirement (2) is not met by the two others.

This may explain the difference between these four acids which the theory of Veldstra (1944) has failed to do convincingly and which apparently cannot be explained either by the principles of Wain et coll. (Fawcett et al. 1953, 1955).

In order to test the validity of this theory some further naphthalene derivatives have been studied. They have been placed at our disposal through the courtesy of Prof. A. Fredga, Uppsala, Dr. Åke Jönsson, Stockholm, and Dr. D. Woodcock, Long Ashton. — It should be mentioned at once that the results with root elongation have confirmed Jönsson's theory, but that a

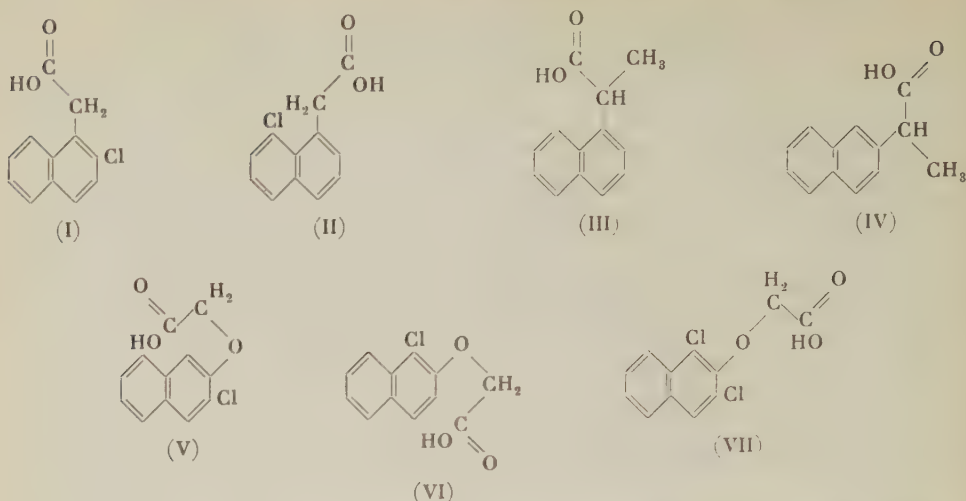


Figure 1. *Approximate sterical formulae of the seven naphthalene compounds tested.* — No attempt is made to indicate the orientation of the carboxyl perpendicular to the ring system, — (I) 2-chloro-1-naphthyl-acetic acid, (II) 8-chloro-1-naphthyl-acetic acid, (III) *racem*- α -(1-naphthyl)-propionic acid, (IV) *racem*- α -(2-naphthyl)-propionic acid, (V) 3-chloro-2-naphthoxy-acetic acid, (VI) 1-chloro-2-naphthoxy-acetic acid, (VII) 1,3-dichloro-2-naphthoxy-acetic acid.

comparison between the root and shoot growth activity of the compounds has led to some rather unexpected results.

The substances tested have been (cf. Figure 1): —

(I) 2-chloro-1-naphthyl-acetic acid (Jönsson), which ought to fulfil the mentioned requirements of an auxin,

(II) its isomer, 8-chloro-1-naphthyl-acetic acid (Jönsson), which ought to be inactive as substitution in the 8-position would prevent the side chain from reaching the centre of the ring system,

(III) *racemic* α -(1-naphthyl)-propionic acid (Fredga), which should be an auxin,

(IV) *racemic* α -(2-naphthyl)-propionic acid (Fredga), which should be inactive according to Jönsson. The effects of (III) and (IV) should be the same as those of the corresponding acetic acids (Burström 1955).

Furthermore,

(V) 3-chloro-2-naphthoxy-acetic acid (Woodcock), which should have auxin action as the unchlorinated acid,

(VI) 1-chloro-2-naphthoxy-acetic acid (Jönsson), in which the formation of a pseudo-ring should be prevented by the chlorine and which ought to be inactive, and

(VII) *1,3-dichloro-2-naphthoxy-acetic acid* (Jönsson), which should be inactive as the analogous di-*ortho*-substituted phenoxy-acetic acids.

Some supplementary tests have been carried out with 2-naphthoxy-acetic acid (2-NOAA), α -(3-indolyl)-isobutyric acid (α -3-IIBA), and combinations of the above-mentioned acids with 3-indolyl-acetic (3-IAA) and isobutyric acids.

The compounds have been tested in our routine wheat root micro test (Burstrom 1955 a) and a three-day root test (Hansen 1954) both with determination of the cell elongation, and some compounds also in an *Avena* cylinder test. In the last mentioned test the mean error of the individual growth determinations amounted to ± 0.03 mm.

Results

(I) — *2-Chloro-1-naphthyl-acetic acid*. — This acid behaves as a regular auxin and its action cannot be distinguished from that of the unchlorinated acid (Table 1); it is less active but does not differ histologically. Thus chlorination in the 2-position does not appreciably change the auxin activity.

(II) — *8-Chloro-1-naphthyl-acetic acid*. — This acid decreases the root growth in higher concentrations but much more slowly than (I), and the question arises whether this is an auxin activity or not. The following considerations are pertinent to this question.

(1) the roots inhibited by (II) do not resemble auxin-treated roots morphologically or histologically; they do not, or do not regularly show the much stimulated root hair growth characteristic of an auxin action. An inhibition by an auxin such as (I) depends mainly upon a reduction in the cell length

Table 1. A comparison between the root growth activity of 1-naphthyl-acetic acid (1-NAA), 2-chloro-1-naphthyl-acetic acid (2-Cl-1-NAA), and 8-chloro-1-naphthyl-acetic acid (8-Cl-1-NAA). — Relative values per cent' of control; cell length of control 185 μ .

Conc. M	1-NAA		2-Cl-1-NAA		8-Cl-1-NAA	
	cell length	cell number	cell length	cell number	cell length	cell number
10 ⁻⁸	99	86	115	98	100	100
10 ⁻⁷	72	65	91	105	100	100
10 ⁻⁶	25 ¹	86	40 ¹	102	76	94
10 ⁻⁵	×	0	23	26	52	68
10 ⁻⁴	×	×	×	0	26 ²	38
10 ⁻³	×	×	×	×	×	0

¹ Excessive root hair formation characteristic of auxin inhibited roots.

² Roots living but no excessive formation of root hairs.

Table 2. *The interaction between 8-Chloro-1-naphthyl-acetic acid (8-Cl-1-NAA) and α -(3-indolyl)-isobutyric acid (α -3-IIBA). — Individual experiments in order carried out.*

8-Cl-1-NAA <i>M</i>	α -3-IIBA <i>M</i>	Cell lengths μ			
0	0	183	187	185	
	3.10^{-7}	266	253		
	10^{-6}	324	318		
	3.10^{-6}	294	344		
	10^{-5}	300	322	322	
10^{-6}	0	172	161		
	3.10^{-7}	278	256		
	10^{-6}	268	242		
	3.10^{-6}	270	283		
	10^{-5}	329	345		
10^{-5}	0	109	84	108	98
	3.10^{-7}	84	96	177	140
	10^{-6}	211	128	94	184
	3.10^{-6}	238	86	204	237
	10^{-5}	275	303	287	135
3.10^{-5}	0	95	108		
	3.10^{-7}	94	136		
	10^{-6}	190	133		
	3.10^{-6}	147	171	124	
	10^{-5}	241	244		

and to a lesser extent upon a reduced rate of cell multiplication. With (II) both properties follow each other closely, which resembles toxic effects of non-auxinic nature.

(2) The acid has been combined with an antiauxin (α -3-IIBA). The results are presented in Table 2. It shows a phenomenon seldom encountered in these tests, namely a pronounced lack of reproducibility of the results, particularly obvious at 10^{-5} *M* of (II). The antiauxin counteracts in a way (II), but at every concentration some tests are found with an elongation near the minimum values. There is also an unusually large variation within each test; the standard deviation $\sqrt{d^2/n}$ normally amounting to ca. 25 per cent has risen to over 45 per cent. This means that the elongation shifts between two extremes, which should be expected if in these combinations there were all-or-none effects of the two acids, either one or the other predominating. The significance of this is not clear.

(3) The result of cylinder tests are shown in Figure 2. The acid is slightly active; the deviations around the zero line are small but significant, indicating a growth inhibition at the lowest concentration and a slight growth promotion at higher ones. If this is an auxin action, the activity of the acid is 0.1 per cent of that of the unchlorinated acid, also shown in the figure. A contamination of that magnitude would suffice for explaining the promoting effect. Never-

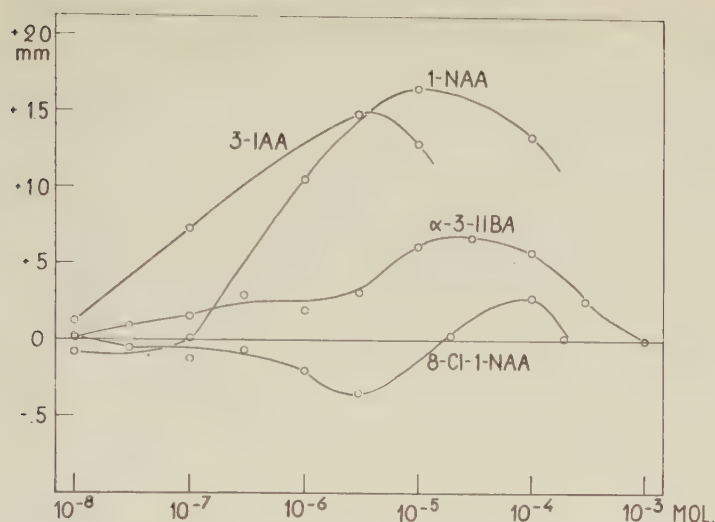


Figure 2. *Avena cylindrica* tests. — Compounds: 3-indolyl-acetic acid (3-IAA), 1-naphthyl-acetic acid (1-NAA), α -(3-indolyl)isobutyric acid (α -3-IIBA), and 8-chloro-1-naphthyl-acetic acid (8-Cl-1-NAA).

theless, another preparation which theoretically could not contain the unchlorinated acid gave exactly the same result. The activity curve with its two different actions is unusual, and at least one of these is not an auxin action. — It can be concluded that the acid owing to the chlorination in the 8-position has lost most, if not all auxin activity. This would be in accordance with Jönsson's expectations.

(III) — *racem- α -(1-Naphthyl)-propionic acid*. — This acid behaves as an auxin on roots in the same manner as 1-naphthyl-acetic acid (Burström 1955). The morphological appearance is also the same but (III) is about one tenth as active as the corresponding acetic acid giving a 50 per cent inhibition at $2 \cdot 10^{-6}$ M.

(IV) — *racem- α -(2-Naphthyl)-propionic acid*. — This acid exerts an action on roots similar to that of the corresponding acetic acid (Burström 1955 a). A slight but significant inhibition is found at very low concentrations amounting to ca. 10 per cent at 10^{-7} M, followed by an increase in root extension. The latter attains a maximum of ca. 25 per cent at 10^{-5} M. This promotion sets in at the same concentration as the inhibition by the homologous 1-substituted propionic acid. It is in accordance with the general rule found with the naphthalene derivatives, that the actions of the corresponding 1- and 2-derivatives are equally strong but of the opposite sign. In the highest concentration the growth falls rapidly down to zero, the usual toxic action

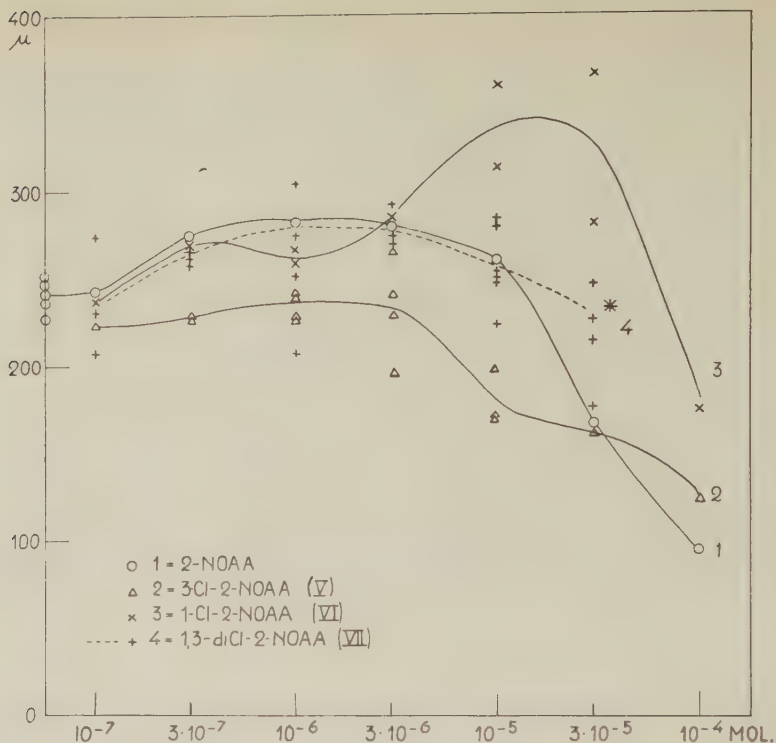


Figure 3. Root cell elongation tests, 24 hours. — Compounds: 1 2-naphthoxy-acetic acid, 2 3-chloro-2-naphthoxy-acetic acid (V), 3 1-chloro-2-naphthoxy-acetic acid (VI), 4 1,3-dichloro-2-naphthoxy-acetic acid (VII). *Roots dying.

not specifically affecting the cell elongation. — The similarity between the 2-naphthyl-acetic and α -propionic acids is in accordance with Jönsson's rules.

(V–VII) — *The 2-naphthoxyacetic acids.* — The results with the unchlorinated acid (2-NOAA) and the three chlorinated acids are presented in Figures 3 to 6; the root cell elongation activity up to 24 hours in Figure 3, the time factor in Figure 4, the interaction with 3-IAA and α -3-IIBA in Figure 5, and the activities in the cylinder test in Figure 6.

The root elongation activity of 2-NOAA was described in a previous paper (Burström 1955 a). It was found to exert three actions: at the lowest concentrations a slight growth depression, followed by a promotion and finally a strong inhibition interpreted as the genuine auxin effect. In the cell elongation (Figure 3) only the two last-mentioned effects show up, but the first is absent; it is obviously not connected with cell elongation.

The first positive effect returns in the 1-Cl and 1,3-diCl derivatives (VI)

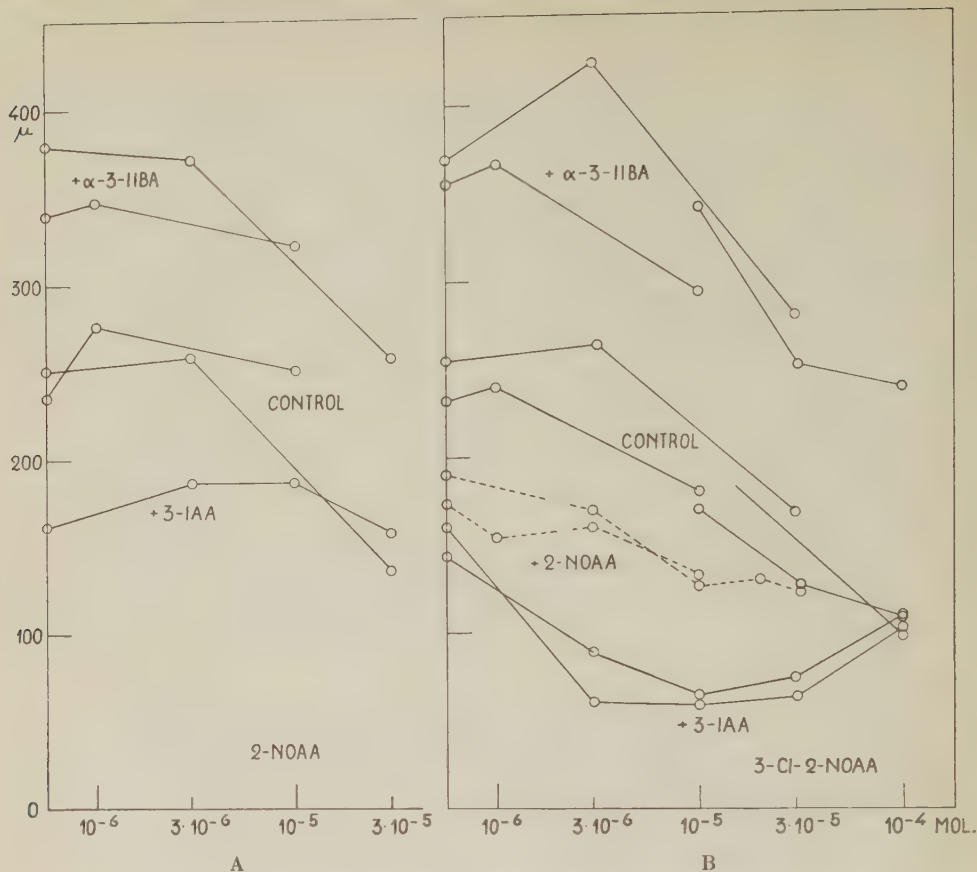


Figure 5. Root cell elongation tests; the interaction of 2-naphthoxy-acetic acids with 3-indolyl-acetic acid (3-IAA) and α -(3-indolyl-isobutyric acid (α -3-IIBA). Concentrations of the additions 3-IAA 10^{-8} M, α -3-IIBA 10^{-5} M, 2-NOAA when added to the other acids (dotted curves) $3 \cdot 10^{-5}$ M.

Figure 5 A. 2-naphthoxy-acetic acid.

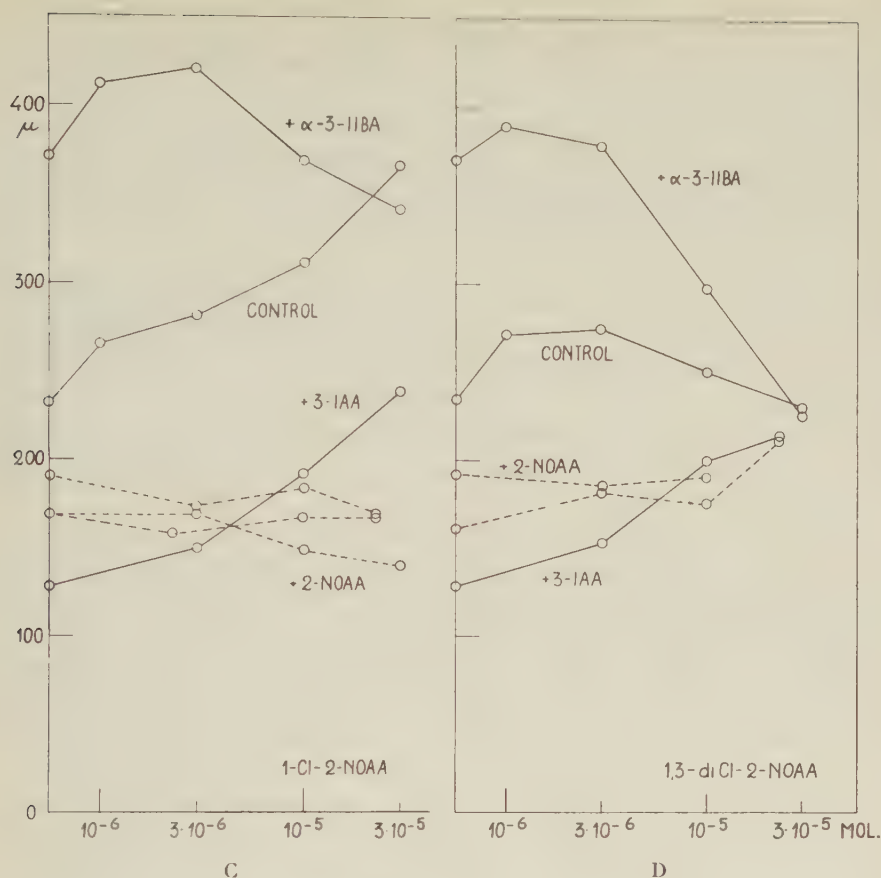
5 B. 3-chloro-2-naphthoxy-acetic acid (V).

5 C. 1-chloro-2-naphthoxy-acetic acid (VI).

5 D. 1,3-dichloro-2-naphthoxy-acetic acid (VII).

According to this test (V) might be called an auxin, (VI) an antiauxin or root auxin, and (VII) inactive. It must be emphasized again, however, that the first positive effect — for the sake of simplicity called the *low-level action* — is independent of the auxinic properties of the acids.

The time factor (Figure 4) is of a certain importance for the outcome of the growth effects. With (VI) and (VII) extension of the duration of the tests



up to 72 hours causes nothing but an earlier onset of the toxicity, but with (V) and 2-NOAA the activity curves change their shape completely. First of all the depression of the elongation — tentatively identified as an auxin action — appears in solutions one tenth as concentrated, and this causes the low-level effects to disappear; they are apparently covered by the auxin actions. Instead a recovery appears at the highest concentration, so that the activity curves pass a deep minimum, a phenomenon previously observed with naphthalene derivatives on roots by Hansen (1954) and by Åberg and Khalil (1953) on coleoptiles.

The interaction with 3-IAA and α-3-IIBA (Figure 5, A–D) has been studied only above 10⁻⁶ M, i.e., the low-level effect has been left out of the picture.

The mode of interpretation used has been that more or less strictly parallel curves of activity of a substance with and without the addition of an indole-derivative indicates a physiologically independent, additive action (Burström

1955 a), whereas if the curves converge and meet, this should imply a similar or antagonistic action, one compound replacing or counteracting the other in one physiological system.

It then appears with 2-NOAA that it is independent of α -3-IIBA but similar to 3-IAA. It has been shown, however (Burström and Hansen), that 3-IAA and α -3-IIBA act physiologically independently, and the logical conclusion to be drawn is that 2-NOAA in these respects behaves as 3-IAA or a genuine auxin on roots.

This is essentially true also of (V) (Figure 5 B). One striking difference is found in combinations with 3-IAA. At medium concentrations the inhibition is stronger than the sum of those of each compound alone, but at the highest concentration a recovery is found. The higher inhibition of (V) should be compared with the peculiar hump of the activity curve of pure (V) in Figure 3, and raises the suspicion whether 2-NOAA and (V) have identical action in all details.

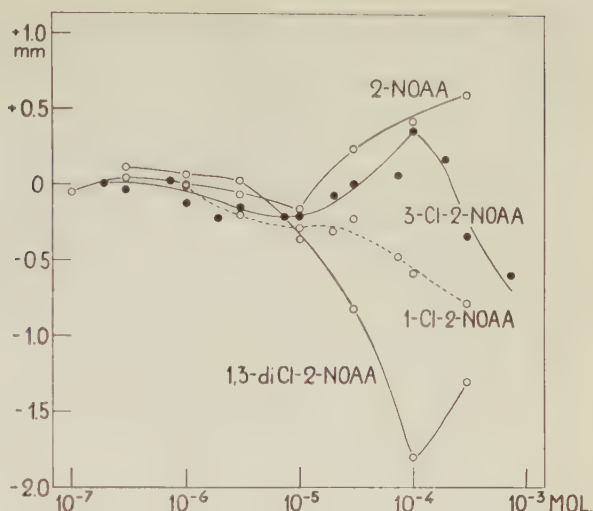
Substance (VI) in Figure 5 C is decidedly an antiauxin or root auxin, acting additively with 3-IAA and similarly as α -3-IIBA. Superficially the two root auxins counteract each other, which only means that together they are supplied in supraoptimal amounts; the absolute upper limit of cell elongation lies slightly above 400 μ , which is reached with these substances. The unchlorinated acid is superimposed on (VI), so that its inhibitory action is not influenced by the presence of (VI) at all. This might imply an unexpected difference between 2-NOAA and 3-IAA, but it must be considered that 2-NOAA is supplied in a 3,000 times higher concentration than IAA, which might be of importance for the outcome of an interaction also involving uptake and migration of the compounds. — Acid (VII), finally, is again of another nature, in itself inactive it annihilates the action of both indole derivatives. It cannot be classified as belonging to either auxins or anti-auxins, but as an antagonist against externally added growth substances.

The results of the *cylinder elongation tests* are presented in Figure 6. All naphthoxyacetic acids start with a slight but statistically significant inhibition of the elongation up to 10^{-5} M. It is interesting to note that this is the reversal of the low-level action on roots, indicating that *the 2-naphthoxyacetic acids in low concentrations decrease the coleoptile elongation and enhance the root cell elongation, and that this is independent of ensuing auxin or auxin-antagonistic effects.*

Above 10^{-5} M 2-NOAA and (V) appear as growth-promoting auxins, (VI) is slightly inhibiting, and (VII) strongly so with a marked recovery at the highest concentration.

According to the usual way of interpreting cylinder tests (VI) and (VII) should be tentatively called 'antiauxins'. However, as shown in Figure 2,

Figure 6. *Avena cylindrica* tests.
— Compounds: 2-naphthoxy-acetic acid (2-NOAA), 3-chloro-2-naphthoxy-acetic acid (3-Cl-2-NOAA), 1-chloro-2-naphthoxy-acetic acid (1-Cl-2-NOAA), and 1,3-dichloro-2-naphthoxy-acetic acid (1,3-diCl-2-NOAA).



α -3-IIBA, which should be an ideal antiauxin, promotes coleoptile elongation, corroborating identical results of Fawcett et al. (1955). McRae and Bonner (1953) have shown, on the other hand, that phenoxyisobutyric acids act as competitors of exogenous auxins, and Ingestad (1953) and Fransson and Ingestad (1955) have found that they compete with native auxin in coleoptiles.

Discussion

The diagnosed actions of the compounds have been summarized in Table 3, which requires some comments. The table is incomplete, in that cylinder tests are lacking in some instances which were judged to be less important.

As 'medium' concentrations have been chosen the ranges of concentration in which a genuine auxin effect appears, or the same range for related compounds. This is, of course, rather arbitrary, and it is hardly justified to assume that all effects entered under 'low' and 'high' levels respectively are strictly comparable. However, it must be emphasized that it is only a question of cell elongation effects. Nevertheless, it is obvious that the compounds exert actions not only of auxinic but also of non-auxinic nature. According to the current opinion an auxin should increase the extension of coleoptiles and decrease that of roots. In this sense and according to the combined tests with 3-IAA and α -3-IIBA the following acids should be classified as auxins: 2-chloro-1-naphthyl-acetic acid, *racem*- α -(1-naphthyl)-propionic acid, 2-naphthoxy-acetic acid, and 3-chloro-2-naphthoxy-acetic acid. The remaining acids offer some difficulties. Three have been called 'root auxins' in the table.

Table 3. *Survey of the action of the compounds investigated.* Effects are given as increases (+) or decreases (—) of cell elongation at three concentration levels for roots and coleoptiles separately. A dot indicates that no tests have been carried out. — Tox.= unspecific toxicity; Rec.= 'recovery' phenomenon.

Compound	Root at levels			Coleoptile at levels			Diagnosis
	low	medium	high	low	medium	high	
3-IAA	—	—	0 ¹	+	—	auxin
α -3-IIBA	0	+	—	+	+	—	'root auxin'
(I) 2-Cl-1-NAA	0	—	tox.	.	.	.	auxin
(II) 8-Cl-1-NAA	0	tox. ²	tox.	—	+	—	no auxin(?)
(III) <i>r</i> -1-NPA	0	—	auxin
(IV) <i>r</i> -2-NPA	—	+	tox.	.	.	.	'root auxin'
2-NOAA	+	—	rec. ²	—	+	.	auxin
(V) 3-Cl-2-NOAA	0	—	rec. ²	—	+	tox.	auxin
(VI) 1-Cl-2-NOAA	+	+	tox.	—	—	.	'root auxin'
(VII) 1,3-diCl-2-NOAA ...	+	0	tox.	—	—	rec.	{inactive antagonist; shoots?

¹ Down to 10^{-11} M. ² in 72 hours' test.

This term has been launched by Hansen (1954) in order to denote compounds increasing *per se* the cell elongation in roots. They are usually called 'antiauxins' but then it must be known that they act by competing with native auxin, and they must then logically by the same virtue decrease the elongation of coleoptiles.

However, this does not hold true of α -(3-indolyl)-isobutyric acid which promotes the coleoptile elongation. Fawcett et al. (1955) have discussed the possibility of a degradation to the acetic acid, finding it highly improbable physiologically. At present we have to reckon with the probability of the indolyl-isobutyric acid itself increasing the elongation in coleoptiles. In this respect 1-chloro-2-naphthoxyacetic acid behaves as should be expected of an antiauxin. According to the root diagnosis its action is identical with that of α -(3-indolyl)-isobutyric acid, but it has the opposite effect on coleoptiles. This is inconsistent with a strict division of the compounds into two groups, auxins and antagonistically acting antiauxins.

Divergences are also found with 8-chloro-1-naphthyl-acetic acid; the inhibition of the root elongation is not of auxin nature and the generally weak effect on coleoptiles is of unknown nature. No less complicated conditions are found with 1,3-dichloro-2-naphthoxy-acetic acid. It is in itself inactive on roots but counteracts both exogenous auxin and 'antiauxin'. Such an antagonism is most easily explained as an unspecific blocking of absorption or transport of the externally applied substances. It inhibits coleoptiles strongly, but if this is an antiauxin action it is incomprehensible why it

does not influence root elongation. The acid resembles structurally the 2,6-substituted phenoxyacetic acids, which were assumed to be inactive until the reverse was shown by Osborne et al. (1954) and Meljnikov et al. (1955). McRae and Bonner (1952) regard them as antiauxins on shoots, but actually demonstrated an antagonism only against external auxin, and Hansen (1954) found them possessing similar unspecific antagonistic actions on roots as the 1,3-chlorinated naphthoxy acid. No thoroughly consistent picture can be drawn at present of the types of activity exerted by these compounds.

Obviously, we cannot rigidly identify every increase of coleoptile elongation with an auxin action, and every increase of root elongation with an antagonistic 'antiauxin' effect. It is also clear that antagonism against externally added compounds can be exerted also by substances in themselves inactive (cf. Hansen, 1954) on the material in question.

An important element in evaluating the activity of compounds is the usually neglected *time factor* (cf. however Audus 1952, McRae and Bonner 1953). Among the naphthoxyacetic acids this comes to the fore in the cumulative nature of auxin inhibitions on roots, less in toxic effects, and not at all in the root auxin actions, as far as the present experiments go. The cumulative nature of the auxin inhibitions has two consequences. — With extended auxin treatment the low-level effects are covered by inhibitions and disappear. It is then impossible without an exhaustive study to show whether an action is an unequivocal auxin effect or if it also comprises other elements. It is also obvious that quantitative comparisons between actions and attempts to formulate activities mathematically are rather hazardous.

It is then hardly surprising that all the formulated *structure-activity theories* have proved to be more or less untenable, unless they are expressed in very general terms (cf. Fawcett et al. 1955). The exceptions are too conspicuous to be neglected. Owing to the obvious heterogeneity of the growth manifestations it is scarcely possible to expect a structure-activity rule to be applicable to more than *one physiologically defined mode of action* and perhaps only within *one group of substances* with similar chemical and physical properties. This would follow from the fact that a measured activity usually includes such elements as absorption, apparently following the same pattern as ion uptake generally (Reinhold 1954), migration, not to mention transformations of a more or less specific nature (Fawcett, Ingram, and Wain 1954, Luckwill and Woodcock 1955). It is thus probably hopeless to look for a master theory covering all cases of structure and modes of activity. In the present instance the occurrence of auxin activity on roots is satisfied by Jönsson's rules (1955). These do not apply to the activity in coleoptiles, as far as can be judged at present; the dubious instances are 8-chloro-1-naphthyl-acetic acid and 1,3-dichloro-2-naphthoxyacetic acid.

All structure-activity theories are founded on the assumption that only 3-indolyl-acetic acid and with it physiologically identical compounds have an activity *per se*, and that in one active system; all others should act by blocking 3-IAA from active or inactivating sites (cf. Gordon 1954). This presumption is, after all, arbitrary. Considering the fact that there may be more than one point of action already in the cell elongation mechanism (Burström 1955 b) and that the actions on root and shoot are not always inverse, it seems appropriate to adopt a less prejudiced view of this question.

Two growth phenomena observed deserve to be specifically mentioned. One is the '*recovery*' phenomenon. It implies that inhibition by a substance passes a minimum and is turned into an increased elongation at incipient toxicity. This is an unusual physiological phenomenon and ought to depend upon specific properties of this type of compounds. It has previously been found with naphthoxyacetic acids on coleoptiles by Åberg and Khalil (1953) and on roots by Hansen (1954). It is impossible to decide whether such a reversion occurs also with growth-promoting effects, because they invariably are followed by toxic effects in high concentrations.

Åberg and Khalil have advanced an interpretation implying that the inhibitors, in their instance 'weak auxins', at first inhibit growth by blocking off endogenous auxin, but in higher concentrations exert an action *per se*. Apart from the fact that such an explanation is at variance with the simple rules of exchange — if such govern the attachment of the auxins — the hypothesis cannot explain the corresponding phenomenon in roots, which are caused by 'strong auxins'.

It is more fruitful to see under what conditions 'recovery' appears. This is on coleoptiles with 1,3-dichloro-2-naphthoxy-acetic acid which is inactive on roots, on roots with the auxins 2-naphthoxy-acetic acid and its 3-chlorine derivative after 72 hours, but after 24 hours only with an addition of 3-indolyl-acetic acid. It is obvious, if this is a uniform phenomenon, (1) that it is connected with inhibitions and not particularly with auxins, (2) that the action is cumulative, and (3) that it increases in the presence of a native auxin. Items (2) and (3) invite a comparison with the well-known adaption of roots to 3-IAA, which may be linked up with the adaptive indolylacetic acid oxidase (Galston and Dahlberg 1954, Siegel and Galston 1955). Yet it is no more than a guess that the explanation could be sought along such lines.

The other special problem is provided by the actions of non-auxinic nature, particularly the *low-level effects*. These appear rather commonly among naphthoxyacetic acids, for the rest their frequency is unknown, since activity curves seldom are followed down to low concentrations. The occurrence is not irregular but involves a growth promotion in roots and an inhibition in coleoptiles. Several examples are found with naphthyl acids (Burström 1955 a).

It is important that the effects are independent of the auxinic or non-auxinic nature in the studied examples, but, nevertheless, are structurally determined cell elongation effects. It would seem as if *they depend mainly upon the type of the ring system*, but not on the ring-chain relationships. This supposition is alien to the current auxin concepts. It is possible that the positive effect on coleoptiles of α -(3-indolyl)-isobutyric acid is no genuine auxin effect either, but something following the indole-system. — In any case it cannot be doubted that this group of compounds exerts cell elongation activities of unknown nature but not identical with the auxin actions.

Summary

A comparison has been made between the cell elongation activity on roots and coleoptiles by eight naphthalene derivatives. The results are summarized in Table 3. — The auxin activity on roots conforms to the rules formulated by Jönsson.

Cell elongation effects — 'low-level effects' — appear in low concentrations of the compounds, which are independent of the genuine auxin actions and obviously governed by the type of ring system present. It has been confirmed that α -(3-indolyl)-isobutyric acid is a growth promotor on coleoptiles; 1,3-dichloro-2-naphthoxy-acetic acid is in itself an inactive antagonist against external growth compounds of both auxin and antiauxin nature. In higher concentrations of certain compounds there appears a 'recovery' phenomenon implying that a strong inhibition of the elongation with increasing concentration or duration of the treatment is reversed.

The implications of the findings for the structure-activity problem of auxins have been discussed.

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References

- Åberg, B. & Khalil, A.: Some notes on the effect of auxin antagonists and synergists upon coleoptile growth. — *Lantbrukshögsk. Ann.* 20: 81. 1953.
Audus, L. J.: The time factor in studies of growth inhibition in excised organ sections. — *J. exper. bot.* 3: 375. 1952.
Burström, H.: Studies on growth and metabolism of roots VI. — *Physiol. Plant.* 4: 470. 1951.
— Evaluation of the growth activity of naphthalene derivatives. — *Physiol. Plant* 8: 174. 1955 a.

- Zur Wirkungsweise chemischer Regulatoren des Wurzelwachstums. — Bot. Not. 108: 400. 1955 b.
- & Hansen, B. A. M.: The mode of growth action of some naphthoxy compounds. — J. exp. bot. (in print).
- Fawcett, C. H., Ingram, J. M. A. & Wain, R. L.: The β -oxidation of ω -phenoxyalkylcarboxylic acids in the flax plant in relation to their plant growth-regulating activity. — Proc. R. Soc. B. 142: 60. 1954.
- Osborne, D. J., Wain, R. L. & Walker, R. D.: Studies on plant growth-regulating substances VI. — Ann. appl. biol. 40: 232. 1953.
- Wain, R. L. & Wightman, F.: Studies on plant growth-regulating substances VIII. — Ann. appl. biol. 43: 342. 1955.
- Fransson, P. & Ingestad, T.: The effect of an antiauxin on the indoleacetic acid content in *Avena coleoptiles*. — Physiol. Plant. 8: 336. 1955.
- Galston, A. W. & Dahlberg, L. Y.: The adaptive formation and physiological significance of indoleacetic oxidase. — Amer. J. Bot. 41: 373. 1954.
- Gordon, S. A.: Occurrence, formation, and inactivation of auxin. — Ann. rev. plant. physiol. 5: 341. 1954.
- Hansen, B. A. M.: A physiological classification of 'shoot auxins' and 'root auxins'. — Bot. Not. 107: 230. 1954.
- Ingestad, T.: Kinetic aspects on the growth-regulating effect of some phenoxy acids. — Physiol. Plant. 6: 796. 1953.
- Jönsson, N. Å.: Synthetic plant hormones VIII. — Svensk kem. tidskr. 67: 166. 1955.
- Luckwill, L. C. & Woodcock, D.: Plant growth regulators I. — J. hort. soc. 30: 109. 1955.
- McRae, D. H. & Bonner, J.: Diortho substituted phenoxyacetic acids as antiauxins. — Plant Physiol. 27: 834. 1952.
- — Chemical structure and antiauxin activity. — Physiol. Plant. 6: 485. 1953.
- Mel'nikov, N. N., Turetskaja, P. H. & Baskakova, J. A.: (Structure and physiological activity of alkyl- and arylphenoxyacetic acids and their derivatives) (transl. from Russian original). — Fiziologija Rastenii 2: 267. 1955.
- Osborne, D. J., Blackman, G. E., Powell, R. G., Sudzuki, F. & Novoa, S.: Growth-regulating activity of certain 2:6-substituted phenoxyacetic acids. — Nature (London) 174: 742. 1954.
- Reinhold, L.: The uptake of indole-3-acetic acid by pea epicotyl segments and carrot discs. — New Phytol. 53: 217. 1954.
- Siegel, S. M. & Galston, A. W.: Peroxidase genesis in plant tissues and its relation to indoleacetic acid destruction. — Arch. bioch. biophys. 54: 102. 1955.
- Street, H. E.: Factors controlling meristematic activity in excised roots VI. Physiol. Plant. 8: 48. 1955.
- Veldstra, H.: Researches on plant growth substances IV. Relations between chemical structure and physiological activity. I. — Enzymologia 11: 97. 1944.